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Evolutionary Dynamics in *Drosophila subobscura*

Analyses of Life History Traits and Microsatellite Loci during Laboratory Adaptation

Pedro Miguel Moraes Corado Simões

DOUTORAMENTO EM BIOLOGIA
(Biologia Evolutiva)

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Tese Orientada por:
Professora Doutora Margarida Matos

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À Minha Família e Amigos

"Biology is the science. Evolution is the concept that makes biology unique."

Jared Diamond

Nota Prévia

Na elaboração da presente dissertação, e nos termos do nº 1 do artigo 40, Capítulo V, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República – II Série Nº 153, de 5 de Junho de 2003, esclarece-se que foram usadas integralmente publicações científicas já publicadas (1), aceites para publicação (1) ou submetidas (3) para publicação, em revistas indexadas de circulação internacional (4) e num livro de editora científica internacional (1), os quais integram os capítulos da presente tese. Tendo os referidos trabalhos sido realizados em colaboração, o candidato esclarece que participou integralmente no planeamento, análise e discussão dos resultados e na elaboração de todos os trabalhos.

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Note: The varying format of the different chapters in this thesis reflects the specific requirements of the scientific publications to which the presented manuscripts have been submitted.

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Resumo

A adaptação a um novo ambiente é um dos principais fenómenos responsável pela complexidade biológica existente. Esta tese de doutoramento propõe-se analisar detalhadamente um processo adaptativo em curso, utilizando uma abordagem experimental. Nesse âmbito, apresenta-se aqui um conjunto de trabalhos que caracteriza detalhadamente o processo de adaptação a um novo ambiente em organismos sexuados. Especificamente, este estudo baseia-se na caracterização de trajectórias evolutivas de caracteristicas fenotípicas (caracteristicas da história da vida, com impacto directo na fitness individual), em populações de *Drosophila subobscura*, desde a sua introdução no ambiente laboratorial. Adicionalmente, alterações genéticas durante a adaptação ao laboratório serão também analisadas recorrendo a marcadores moleculares, especificamente microsatélites.

Esta tese procura responder a diversos aspectos relevantes do fenómeno adaptativo, tais como a caracterização pormenorizada das alterações fenotípicas (de curto e longo prazo) que resultam da introdução de populações num ambiente controlado pelo homem (cativeiro); a possibilidade de existência de fenómenos de convergência relativamente a um estado de equilíbrio evolutivo no novo ambiente; a repetibilidade do processo adaptativo e o impacto de diferenças genéticas de origem no mesmo; a magnitude e dinâmica das alterações genéticas associadas a este processo, bem como eventuais implicações para programas de conservação de diversidade genética, tendo como objectivo uma melhor gestão de populações cativas.

O primeiro capítulo desta tese consiste numa breve introdução à àrea da evolução experimental como abordagem para estudar em detalhe o processo adaptativo de populações, questão central da biologia evolutiva. Serão também abordadas brevemente as noções fundamentais de genética populacional ao nível molecular e as aplicações de técnicas moleculares em estudos de evolução experimental. Finalmente é realizada uma breve revisão crítica dos estudos existentes na àrea específica de adaptação laboratorial e as suas contribuições para a compreensão do processo adaptativo, definindo-se a contribuição específica desta tese para o desenvolvimento da área.

No capítulo 2 é apresentado um estudo detalhado de um processo adaptativo de curto prazo (até 40 gerações no laboratório), bem como analisado o impacto de fundos genéticos distintos no processo adaptativo. Para isso é feita a análise das trajectórias evolutivas em caracteristicas da história da vida de dois conjuntos de populações laboratoriais resultantes de colheitas realizadas em duas localizações geográficas distintas (populações "AR" de Arrábida, Portugal e populações "TW" de Sintra, Portugal).

A resposta evolutiva de longo prazo é também analisada em detalhe, através da análise de diferentes conjuntos de populações laboratoriais, "NW" e "NB", mantidas durante 86 e 176 gerações em laboratório, respectivamente.

O capítulo 3 apresenta um sumário dos dados de adaptação ao laboratório obtidos em caracteristicas fenotípicas para as populações NB, NW, AR e TW, juntamente com uma breve revisão dos trabalhos mais relevantes na área de evolução experimental em *Drosophila* (focando principalmente estudos de adaptação ao laboratório). É também discutido o impacto de diferentes forças - selecção natural, deriva genética, inbreeding... - nos padrões evolutivos obtidos. Adicionalmente, é testada a eficácia do método comparativo (interpopulacional) para inferir as trajectórias evolutivas reais (intrapopulacionais).

No capítulo 4 é estudada a repetibilidade do processo adaptativo, recorrendo a dados obtidos a partir de diversas fundações diferindo quer no espaço (Sintra e Arrábida) quer no tempo (diferentes estudos iniciados em 1998, 2001 e 2005). É também estudada a importância de efeitos de amostragem *vs.* efeitos geográficos ao nível da fundação no processo adaptativo (i.e. análise de 4 fundações sincrones realizadas em 2005). Esta abordagem permite testar a importância de efeitos geográficos e temporais na composição genética das populações e subsequente impacto no processo adaptativo. Será discutida a questão da relevância destes factores de contingência *vs.* repetibilidade do processo de adaptação ao laboratório.

No capítulo 5 é realizada uma caracterização genética (utilizando marcadores moleculares) das populações "NW" na sua geração 49 no laboratório, comparativamente à estrutura genética apresentada por populações recém-troduzidas: populações "TW" na sua 3ª geração no laboratório. Este estudo envolve a genotipagem de 10 microsatélites, focando diversos aspectos da estrutura genética destas populações (variabilidade e diferenciação genética; tamanho efectivo populacional). Os resultados

são discutidos considerando a problemática de conservação de variabilidade genética em populações cativas.

Por último, o capítulo 6 apresenta um detalhado estudo da dinâmica temporal de alterações genéticas durante o processo de adaptação ao laboratório, tendo como base as alterações nas frequências alélicas em microsatélites nas primeiras 40 gerações das populações AR e TW. Assim, diversos parâmetros genéticos das populações AR e TW são obtidos e comparados, nomeadamente a variabilidade genética inicial, a taxa de declínio de variabilidade em cada uma das populações, o tamanho efectivo populacional, etc..., nestas populações evoluindo em síncronia. Os resultados obtidos permitem também uma comparação das alterações genéticas e fenotípicas (ao nível da resposta adaptativa) entre os dois conjuntos de populações. Adicionalmente, e tendo em consideração a história evolutiva das populações laboratoriais, é comparada a diferenciação genética molecular (F_{st}) – baseada em microsatélites – com a diferenciação quantitativa (Q_{st}) – baseada em caracteristicas da história da vida – em cada população AR e TW entre as gerações 3 e 40 em laboratório. A expectativa *a priori* é a de Q_{st} superior a F_{st}, como resultado da acção da selecção natural sobre caracteristicas fenotípicas (quantitativas) durante o processo de adaptação ao laboratório. Finalmente, utilizando os dados temporais de variabilidade genética em cada uma das populações AR e TW (nas gerações 3, 14 e 40 no laboratório) é aplicado um teste à existência de arrastamento selectivo ('selective sweep') em algum dos microsatélites analisados.

Os resultados obtidos no conjunto destes estudos evidenciaram uma clara resposta evolutiva das populações como resultado da adaptação a um novo ambiente (laboratório). Esta resposta evolutiva caracterizou-se por um aumento de desempenho nas características fenotípicas associadas à fecundidade (como a idade da primeira reprodução e fecundidades jovem e de pico) para as diferentes populações analisadas (capítulos 2, 3 e 4). Verificou-se também que a taxa evolutiva apresentava uma associação positiva com a diferenciação fenotípica inicial relativamente a um estado de equilibrio evolutivo no ambiente laboratorial (capítulo 2).

Da análise das trajectórias evolutivas resultou uma diferente resposta consoante as características de história da vida consideradas. Apesar de ser expectável uma melhoria generalizada no desempenho em laboratório, principalmente numa primeira fase do processo de adaptação, verificou-se uma ausência de resposta evolutiva

consistente para algumas caracteristicas fenotípicas, especificamente a resistência à inanição, tempo de desenvolvimento e viabilidade juvenil (capítulos 2 e 3). Assim, concluiu-se que no novo ambiente diferentes características contribuem diferencialmente para a fitness, fazendo com que a resposta adaptativa global resulte num padrão diferencial de alterações evolutivas nas diferentes caracteristicas.

Apesar da análise das trajectórias evolutivas no processo inicial de adaptação ao laboratório (primeiras 15-20 gerações) ter revelado uma resposta evolutiva qualitativamente semelhante entre diferentes fundações, a repetibilidade nos padrões adaptativos gerais não teve correspondência em termos de taxa evolutiva. Factores de contingência evolutiva, nomeadamente alterações genéticas associadas a efeitos temporais ou espaciais de amostragem, revelaram um claro impacto nas dinâmicas adaptativas das diferentes populações, promovendo deste modo variabilidade nas respostas observadas (capítulos 2 e 4).

A resposta evolutiva de longo prazo foi também analisada em populações estabelecidas durante dezenas de gerações em laboratório (populações NW e NB). Os resultados obtidos indicam que esta resposta evolutiva está sujeita a um processo de interacção entre duas forças evolutivas opostas, a selecção natural e a deriva genética, a primeira causando erosão progressiva de variabilidade genética, a última causando depressão de consanguinidade. À medida que as populações se adaptam progressivamente a um novo ambiente, as hipóteses de melhoramento no desempenho de determinadas características diminuem, originando um abrandamento da resposta selectiva (*plateau*). Este padrão verificou-se particularmente para as características de fecundidade. Adicionalmente, em características de menor relevância para a *fitness* (como parece ser o caso da resistência à inanição em ambiente laboratorial), efeitos de consanguinidade podem assumir real preponderância, como resultado da acumulação crescente de alelos recessivos de efeitos deletérios no fundo genético, devido à menor acção da selecção natural. Isto ficou demonstrado pelo declínio no desempenho desta característica, observado nas populações NW e NB, em fase mais avançada do processo de adaptação (capítulos 2 e 3).

Da análise comparativa entre as trajectórias evolutivas reais e as inferidas através do método comparativo ficou demonstrada a utilidade deste último para a caracterização de padrões lineares de resposta. Contudo, para características que apresentam menor consistência temporal de alterações (especificamente a resistência à

inanição) o método comparativo não se revelou apropriado para inferir a resposta evolutiva de uma população (capítulo 3).

As análises da estrutura genética molecular das populações laboratoriais indicaram a existência de considerável variabilidade genética. Este resultado verificou-se tanto para as populações NW após 49 gerações em laboratório (capítulo 5) como para as populações AR e TW com 40 gerações (capítulo 6). Contudo, os resultados obtidos indicaram uma perda de variabilidade genética desde a introdução em laboratório, o que é principalmente devido à acção da deriva genética em populações confinadas (capítulos 5 e 6). Esta perda de variabilidade genética foi superior nas primeiras 14 gerações quando comparada com o período posterior (gerações 14 a 40), correspondendo também a um menor efectivo populacional neste periodo inicial. Estes resultados sugerem uma interacção entre a deriva genética e as maiores pressões selectivas associadas às primeiras gerações após introdução no laboratório (capítulo 6).

É também relevante referir que os dois conjuntos de populações evoluindo em sincronia (AR e TW) apresentaram valores bastante semelhantes de variabilidade genética assim como tamanhos efectivos populacionais próximos, apesar de estarem diferenciados genéticamente (capítulo 6).

As análises comparativas de dados fenotípicos e moleculares durante o processo adaptativo permitiram concluir que a variabilidade genética obtida com marcadores moleculares não reflecte de uma forma clara as diferenças entre populações, no que respeita ao seu potencial evolutivo (capítulo 6). De facto, os padrões de variabilidade genética das populações AR e TW apresentaram uma assinalável semelhança, apesar das diferenças nas dinâmicas evolutivas entre estas populações, principalmente na fase inicial do processo adaptativo (capítulos 2 e 4). Deste modo, inferências de potencial evolutivo ou dinâmica adaptativa a partir de dados moleculares devem ser analisadas com reserva. Apesar disso, a análise combinada de informação genética quantitativa (associada a características fenotípicas) e molecular revelou-se útil no sentido de permitir inferências gerais relativamente à história evolutiva das populações. Particularmente, o uso de informação molecular como hipótese nula de neutralidade, permitiu testar a importância relativa de efeitos de deriva genética *vs.* selecção natural para explicar fenómenos de divergência populacional, contrastando a menor diferenciação genética em marcadores neutros relativamente à de características relevantes para a fitness (capítulo 6).

Por último, obtivemos sugestões da ocorrência de um fenómeno de arrastamento selectivo num dos marcadores moleculares utilizados, particularmente durante as primeiras 14 gerações de adaptação ao laboratório (capítulo 6). A confirmar-se este resultado, ele junta-se a um conjunto crescente de estudos que indicam que a selecção natural terá também um impacto nos padrões de variabilidade genética molecular funcionalmente neutra. Este tipo de análise poderá ser de extrema utilidade no sentido de aumentar o conhecimento de genes (ou regiões genómicas) directamente envolvidas em processos de adaptação local a novos ambientes.

De um modo geral, esta tese ilustra o poder da evolução experimental como abordagem para o estudo das complexidades associadas ao fenómeno de adaptação biológica. A evolução experimental serve, em última análise, como uma ferramenta para discernir a importância relativa de diferentes mecanismos evolutivos na produção da diversidade biológica e multiplicidade de respostas evolutivas. Adicionalmente, este estudo demonstra claramente os benefícios resultantes da combinação de uma aproximação experimental tradicional (nomeadamente através de trajectórias evolutivas de características quantitativas) com o recurso a técnicas moleculares. Esta abordagem permite caracterizar de uma forma detalhada e em diversas vertentes as alterações evolutivas, expressas a nível fenotípico e molecular, que ocorrem durante um processo adaptativo a um novo ambiente.

Abstract

This study uses an experimental evolution framework to thoroughly analyse an ongoing evolutionary process. In this thesis I present a detailed study of an adaptive process occurring in *Drosophila subobscura* populations as a result of their introduction in the laboratory environment. Diverse relevant questions are addressed, associated with the evolutionary changes that arise when wild populations are introduced in a controlled environment. Short and long-term evolutionary responses are studied for several adult and juvenile life-history traits through the analysis of real-time evolutionary trajectories. Genetic changes during laboratory adaptation are accessed through the analysis of microsatellite markers. The results obtained allowed the following conclusions: (1) Adaptation to the laboratory comprehended general increased performance in fecundity related traits; (2) Simple correlated responses to adaptation did not prove accurate, illustrated by a consistent absence of directional evolutionary response for both starvation resistance and juvenile traits; (3) General repeatability of evolutionary response across populations studied was found for the early stages of the adaptive process, though the detailed adaptive dynamics (rate of convergence to control populations) differed across foundations; (4) Long-term domestication involved a balance between opposite forces of selection and inbreeding, with the outcome varying according to the trait; (5) Molecular measures of genetic diversity did not reflect accurately differences in the evolutionary potential of populations; (6) The combined use of quantitative and molecular genetic information provided valuable insight on the evolutionary history of populations; and (7) There was a suggestion that natural selection acted on molecular genetic diversity patterns through genetic hitchhiking.

This thesis clearly illustrates the power of experimental evolution to study the complexities of biological adaptation. Furthermore, this work highlights the benefits that can arise from the combination of a traditional experimental evolution approach with molecular techniques for a detail characterization of the phenotypic and genetic changes occurring during adaptation.

Keywords: Evolutionary dynamics; Laboratory Adaptation; Life history traits; Microsatellites; *Drosophila subobscura*.

Chapter 1.
INTRODUCTION

The field of Evolutionary Biology has two major goals: to discover the history of life on earth; and to understand the causal processes of biological evolution. Evolution is fundamental to a complete understanding of biological phenomena occurring at the most diverse levels. Furthermore, it is also extremely valuable for human welfare and societies in general, given its contributions to the improvement of human health, agriculture and renewable resources, natural products and a better understanding of the different areas of human biology (e.g. genetics, physiology, behaviour…).

The study of biological evolution has been pursued using different approaches, such as: phylogenetic inference methods; paleontological information; inferences from genetic patterns; characterization of genetic and phenotypic variation; inferences based on comparative methods; and through experimentation (Meagher & Futuyma, 2001). This thesis is focussed on the last approach referred – the discipline of experimental evolution.

This thesis presents a detailed study of the process of adaptation in *Drosophila subobscura* populations subjected to laboratory culture. This study addresses such questions: as the phenotypic changes that arise when wild populations are introduced to a *human controlled* environment; convergence to a novel evolutionary equilibrium in the new environment; the impact of distinct genetic backgrounds on adaptation; the repeatability of adaptive processes; the magnitude and pace of the genetic changes that occur throughout this process; as well as potential implications for the management of captive populations for conservation purposes.

I will start by briefly explaining the principles of research using experimental evolution. I will then focus on the field of laboratory adaptation, providing a general state of the art in this specific area of research. I will end this Introduction section by explaining the general plan of this dissertation and highlighting the major questions addressed.

1.1 Principles of Experimental Evolution

Experimental evolution is a field of evolutionary biology that studies evolutionary processes through direct observation of the course and consequences of such processes. In such studies, the evolution of an entire population is the basic unit of observation (Rose *et al.,* 1996). The generic structure of experiments in this field involves measurements of the dynamics of evolution in response to a particular selection treatment in replicated populations under controlled conditions (Rose *et al.,* 1996; Chippindale, 2006). The most obvious advantage of laboratory studies of experimental evolution is the possibility that they afford to control, eliminate, or unveil confounding factors (Rose *et al.,* 1996). A wide variety of problems can be addressed with this experimental approach: the repeatability of adaptive patterns, the importance of constraints on genetic evolution, etc (Rose *et al.,* 1996; Prasad & Joshi, 2003; Chippindale, 2006). It is important to state clearly that the aim of experimental evolution studies is not to mimic or infer the evolution of wild populations, but instead to explore a wide range of evolutionary outcomes and mechanisms.

Experimental evolution studies are commonly divided into two different types: artificial selection, and laboratory natural selection. In artificial selection, the experimenter plays an active role by deliberately choosing the individuals that will contribute to the next generation. On the other hand, with laboratory natural selection, populations are handled according following a general procedure, but are otherwise allowed to evolve without further intervention from the researcher (Garland, 2003; Fuller *et al.,* 2005). Laboratory natural selection has been increasingly used over artificial selection to tackle adaptive processes. Among other differences, it uses much larger population sizes than artificial selection, thus allowing selection to predominate over genetic drift in population differentiation (Chippindale, 2006). Experimental evolution studies have also been performed in wild populations (e.g. Endler, 1986; Losos *et al.,* 1997, 2004; see Reznick & Ghalambor, 2005 for a review), although on a much reduced scale. This is due to the difficulty of achieving adequate experimental control in studies conducted in the wild. Such difficulties include the higher number of uncontrolled (and potentially confounding) variables that may influence evolutionary processes and also the difficulty of having proper replication and appropriate control populations (Rose *et al.,* 1996).

I will briefly address the most important methodological aspects of experimental evolution: I) Organism of study; II) Experimental design; III) Controls; IV) Replication.

The organism of study is chosen according to the aim of the experiment (e.g. evolution in sexual *vs.* asexual organisms). Relevant issues for the choice of experimental organism include generation time, size, manipulability, etc… The most commonly used organisms are *Escherischia coli* – an asexual organism – and *Drosophila melanogaster* – a sexual organism. *Drosophila* is an exceptional model given that it allows testing of evolutionary responses based on existing genetic variance on a reasonable timescale, being a sexual organism with a fast generation time. Furthermore, the increasing knowledge of *Drosophila* physiology, ontogenetics, cytogenetics and molecular biology has contributed to a better understanding of the integrated functioning of sexual organisms as a whole, therefore allowing stronger inferences regarding the mechanistic foundations of evolutionary processes.

An experimental evolution design should rule out potential confounding factors that might obscure the association between the selection treatment that is imposed and the evolutionary response that is observed. Thus, the effect of a particular selection regime should be addressed by comparing the experimental populations subjected to this regime with another set of populations, used as control, that differs from the former solely in the absence of the particular treatment imposed by the researcher. This is not easy to achieve, since these studies focus on dynamic biological entities that can be differently affected by a wide variety of factors (e.g. inbreeding, genotype-by-environment interactions). To minimize potential effects besides the ones being tested, maintenance procedures and population sizes should be similar in both control and selected populations in order to avoid inadvertent selection or differential inbreeding effects. This will reduce the importance of confounded selection mechanisms, genetic drift, and other potential sources of artefact affecting the evolutionary responses observed (Rose *et al.,* 1996; Garland, 2003; Chippindale, 2006).

The use of replication – several populations derived from a common ancestral and subjected to the same treatment – both in the experimental and control regime is essential in experimental evolution studies. This design feature limits the impact of genetic drift, as well as drift-selection interactions, since the variation between replicate populations within each regime (both selected and control) can be subsequently removed from the analysis. Replication is particularly crucial in tests of general evolutionary predictions (Rose *et al.,* 1996). Obviously, with these issues of scientific

inference in mind, the number of replicate populations has a major influence on the statistical power of the experiment.

I will now provide some illustrative examples of the potential and scope of experimental evolution studies.

The long-term experimental evolution studies in *Escherichia coli* by Lenski and his collaborators have been particularly important in the study of long-term adaptation (e.g. Lenski *et al.,* 1991; Lenski, 2004). These studies rely on a very simple experimental design: populations of *E. coli* derived from a single cell are allowed to evolve in a constant, controlled environment. Several aspects of this design are important (see Lenski, 2004): the high number of replicate populations studied (12), all derived from one ancestral cell; the opportunity to compare directly experimental populations with their (previously frozen) ancestor strain, which provides an excellent measure of the initial performance of the experimental populations; the ability to use an exceptionally high number of individuals per population ($5x10^8$ cells per population); and also the possibility to study adaptation over a large number of generations, given the rapid generation time (around 2400 per year). The fact that these populations start with low genetic variability, both within and between populations, and also the timescale of these studies in microorganisms facilitates test of the impact of spontaneous new mutations on the initial adaptive response and also the mutation-selection balance in a long-term perspective.

Throughout the experiment of Lenski and collaborators, bacterial populations were maintained in a liquid, buffered, minimal-salts medium supplemented with glucose as the sole source of carbon and energy (Lenski *et al.,* 1991). In the first 2000 generations a clear response was observed, with an increase of fitness of around 37% (Lenski *et al.,* 1991). Interestingly, the individual fitness trajectories for each population during this period present a step-function as a result of the spread of a beneficial mutation in the population (Lenski & Travisano, 1994; Lenski, 2004), clearly reflecting the importance of mutation events in the adaptive response of asexual populations.

After this rapid evolutionary response, the increase in fitness slowed down, reaching a 70% increase after 20000 generations (Cooper & Lenski, 2000; Lenski, 2004). In fact, between 15000 and 20000 generations, the rate of improvement relative to the ancestor was only about one tenth of the average rate in the first 5000 generations

(Cooper & Lenski, 2000). This deceleration of the evolutionary response through time clearly indicates that populations are reaching an adaptive peak (Lenski, 2004).

It is also noteworthy that *E.coli* replicate populations presented similar evolutionary patterns in general, not only in fitness but also in diverse features of their biology such as physiology and cell morphology. Evidence for mutation events with pleiotropic effects was also found in this adaptive process, with improved performance on glucose medium in conjunction with reductions in other catabolic functions (Cooper & Lenski, 2000; Elena & Lenski, 2003; Lenski, 2004).

The evolutionary dynamics of asexual bacteria can however be quite different from those of sexual organisms. Indeed, recombination in sexual organisms generates considerable genetic variation in each population, allowing natural selection to "work" on this standing genetic variation. Thus, the adaptive patterns presented by sexual organisms will depend much more on both standing genetic variation and recombination events than on the mutational input of new allelic variants (see Levin & Bergstrom, 2000).

Contrary to the situation with asexual organisms, relative fitness is not easily measured in sexual organisms. As such, studies in sexual organisms often use surrogates of fitness, mainly life-history traits. These traits play an important role in the life-cycle and influence directly the reproductive success of organisms (e.g. age of maturity, number of offspring, life span,…) and therefore are expected to have strong effects on fitness (Stearns & Hoekstra, 2000).

Life-history traits generally have a polygenic basis that causes phenotypes to vary along a continuum as a result of the effects of multiple loci (Falconer & Mackay, 1996). Traits with such a complex genetic basis and continuous variation are called quantitative traits and their study and description relies on statistical parameters such as the variance of individuals within a population. The phenotypic variation of a particular trait may have two major causes: genetic differences between the organisms and differences in the environment (genetic and environmental variation). The part of the genetic variation that results in response to selection is termed the additive genetic variation. Additive effects correspond, at a statistical and population level, to the part of the deviations from the average population value that are numerically additive among alleles. These effects are directly related with the heritability (similarity between parents and offspring) of the trait. Other sources of genetic variance exist: dominance interactions between alleles at a particular locus and epistatic interactions between

alleles at different loci. Finally, interactions between the genotype and the environment (I_{gxe}) may also have an important effect in shaping the expression of life-history traits (Falconer & Mackay, 1996; Lynch & Walsh, 1998).

One of the most noticeable experimental evolution studies in a sexual organism is the Irvine *Drosophila* experimental evolution system (see Rose *et al.,* 2004). Several major topics of evolutionary biology have been addressed by this team such as the evolution of ageing, reverse evolution, the evolution of late life plateaus, etc...These and other topics have been accessed by the study of the evolutionary response of several life-history traits to different selection regimes (e.g. early and late fecundity, starvation resistance, accelerated development time…). This system involves useful design features such as: *i)* considerable population sizes (around 1000 or more), *ii)* 5 replicate populations per selection regime; *iii)* pairing of each selection regime with controls; *iv)* implementation of selection regimes after adaptation to the laboratory environment (see further details in Rose *et al.,* 2004). These studies have shown that laboratory populations of moderate size have considerable potential for evolutionary response: almost all traits subjected to direct selection increased in performance (see Rose *et al.,* 2004). Furthermore, evidence was found for genetic trade-offs between relevant fitness traits such as early fecundity and longevity due to antagonistic pleiotropy (Rose, 1984; Rose *et al.,* 2004). Antagonistic pleiotropy occurs when an allele confers beneficial effects to a given trait (e.g. early fecundity) at the cost of deterioration in others (e.g. longevity).

These studies also highlight the specificity of life-history evolution, given that adaptive responses may vary considerably, accordingly to the environment in which experiments are performed (Leroi *et al.,* 1994a; Rose *et al.,* 2005). For example, Leroi *et al.* (1994a) found that genotype-by-environment interactions "masked" the underlying genetic correlation between early and late fecundity previously observed in other studies (e.g. Rose, 1984), as a consequence of differences between standard and assay environments as well as slight inadvertent differences in maintenance regimes. In fact, life-history evolution (and genetic correlations, in particular) has proven to be extremely dependent on genetic background, inbreeding, and environmental effects, rendering predictions of future adaptive patterns extremely difficult. According to Rose *et al.* (2005), experimental evolution studies should concentrate on specific testable predictions and the genetic mechanisms underlying the (un)predicted genetic responses.

This is the case of the reverse evolution experiment performed by Teotónio & Rose (2000), also using the Irvine system. In this experiment, several populations subjected to different selective regimes "returned" to the same selective regime of their ancestral population. The reversibility of evolution was defined as a clear expectation despite possible constrains to adaptive evolution (e.g. lack of additive genetic variation, pleiotropic effects and/or epistatic interactions). Reversion was found to occur but different patterns were obtained (i.e. from fast and complete to slow, incomplete reversion during the 50 generations of the study), depending on the prior evolutionary history of the populations and the traits analysed. Lack of genetic variance and epistatic effects did not limit reverse evolution (Teotónio & Rose, 2000; Teotónio *et al.,* 2002).

Several other studies of laboratory natural selection in *Drosophila* have also contributed to a better understanding of adaptive evolution (see Bell, 1997; Prasad & Joshi, 2003, for an extensive review). These studies have addressed the effects of different densities (e.g. Mueller *et al.,* 1991; 1993; Sokolowski *et al.,* 1997), different temperatures (e.g. Huey *et al.,* 1991; Santos *et al.,* 2004, 2005), demographic regimes (e.g. see Luckinbill *et al.,* 1984; Rose, 1984; Partridge & Fowler, 1992; Leroi *et al.,* 1994a; Nunney, 1996; Chippindale *et al.,* 1997), several stresses (e.g. Rose *et al.,* 1992; Chippindale *et al.,* 1996; Harshman *et al.,* 1999; Bettencourt *et al.,* 2002; Folk & Bradley, 2005; Baldal *et al.,* 2006), among others.

Clear responses to direct selection have been observed in most of these studies for almost all functional characters tested, thus indicating reasonably abundant genetic variation in most populations. Trade-offs appear to be relatively common in life-history evolution, as revealed by the decline of functional characters that are not the target of selection, as opposite to an increase in more relevant fitness traits (see Bell, 1997; Prasad & Joshi, 2003 for examples). However, as mentioned above, these genetic correlations are dependent on the specific environment of the populations, appearing and disappearing as a result of minor environment changes (Rose *et al.,* 1996; Chippindale *et al.,* 2003; Prasad & Joshi, 2003; Rose *et al.,* 2005). Indeed, different environments can alter the relative importance of different traits to overall fitness, rendering fitness clearly context-specific (Prasad & Joshi, 2003).

Overall, these studies of laboratory natural selection in *Drosophila* have highlighted the complexity of the evolutionary process. Evolutionary responses can be influenced by I_{gxe}, inbreeding, pleiotropic or epistatic effects, and prior evolutionary history, each capable of producing substantial deviations from *a priori* expectations.

Furthermore, functional traits can evolve differently across populations even when fitness shows a more uniform evolutionary outcome (e.g. see Cohan & Hoffmann, 1989; Teotónio & Rose, 2001; Prasad & Joshi, 2003). Experimental evolution in general, and laboratory natural selection studies in particular, by defining particular testable variables and reducing "noise" effects, are the best tool for unravelling the complexities of potential adaptive processes and their underlying mechanisms (see also Rose *et al.,* 2004; Chippindale, 2006). I will now concentrate on a particular kind of laboratory natural selection study, the study of adaptation to the laboratory environment *per se*, which is the primary concern of my dissertation.

1.2 Laboratory Adaptation

Adaptation is a fundamental concept in evolutionary biology. The detailed study of adaptation as a process is therefore of crucial importance to a better understanding of biological evolution and conservation. As explained above, this is the main focus of laboratory natural selection studies. In these studies, populations typically *adapt* to the particular conditions imposed by each selective regime (Rose *et al.,* 1996).

Populations may differ in their specific rates of adaptation as well as in their final evolutionary outcome. How much populations converge when adapting to the same environment remains a central issue in evolutionary biology. The repeatability of evolutionary patterns has been a prominent topic of research in experimental evolution, often associated with the role of history and chance in adaptive evolution (e.g. Cohan, 1984a,b; Cohan & Hoffmann, 1989; Travisano *et al.,* 1995; Teotónio & Rose, 2000; Teotónio *et al.,* 2002; Joshi *et al.,* 2003).

One powerful approach to studying these issues is through the detailed analysis of the evolutionary dynamics of populations colonizing a novel environment. A new environment contains a multitude of different challenges to which populations will likely respond. In laboratory adaptation studies, the laboratory is this new environment (Matos *et al.,* 2000a). Laboratory populations are subject to multiple changes relative to their circumstances in wild environments. These include changes in demographic structure, population size and also a reduction of potentially stressful factors, such as predation and inter-specific competition. Maintenance in a controlled environment also leads to changes in several abiotic factors, such as temperature, availability and quality of nutrients, space, etc…. Under these new conditions, genotypes that are better adapted

– e.g. with respect to the acquisition of resources - may exhibit generally better performance in several life-history traits relative to less adapted ones, producing positive genetic correlations among functional traits during the early phases of culture in the novel environment. As adaptation proceeds, the genetic variance associated with such positive pleiotropy will be eroded, and genetic correlations may shift to negative values (Service & Rose, 1985; Chippindale, 2006). As a consequence of the evolution of this negative genetic correlation between functional traits – a genetic trade-off, less relevant fitness traits may later drop in performance. The evolutionary dynamics of different traits may thus have a complex pattern, as a function of these shifts in genetic architecture during the adaptive process. Moreover, relaxed selection may also occur in less relevant traits, potentially leading to a decrease in their performance during long-term laboratory culture, due to the increase in frequency of partially recessive deleterious alleles originally present at low frequencies in the population (Bryant & Reed, 1999; Charlesworth & Charlesworth, 1999).

Genetic variation is expected to be most abundant in large natural populations or in laboratory populations at the moment of their foundation from wild samples, since these samples will not have lost genetic variability due to either genetic drift with small population sizes or intense directional selection during domestication. This is one more reason why studies of evolutionary domestication are of particular interest, as they allow us to characterize the evolutionary dynamics of local adaptation in populations with considerable genetic variation at the start of selection.

Thus, the analysis of the colonization and subsequent evolution in the laboratory environment can be an ideal scenario to study adaptive patterns and changes in overall fitness as a result of the new environmental conditions imposed. Important evolutionary phenomena such as convergence to equilibrium states, deceleration of evolutionary response, and evolutionary stasis are thus a preponderant concern of such studies (Matos *et al.,* 2002). These controlled laboratory studies allow inferences as to the genetic mechanisms underlying the adaptive processes and therefore contribute to a better understanding of such evolutionary phenomena.

Few studies of laboratory adaptation in *Drosophila* were performed until the 1990s (e.g. Dobzhansky *et al.,* 1964; Tantawy & El-Helw, 1966, 1970). Since that decade a growing body of data has appeared in the literature (e.g. Matos *et al.,* 2000b, 2002, 2004; Sgrò & Partridge, 2000; Hoffmann *et al.,* 2001; Krebs *et al.,* 2001; Gilligan

& Frankham, 2003; Reed *et al.,* 2003; Griffiths *et al.,* 2005; see chapter 3 for a review of some of these studies).

Most of these studies find a clear evolutionary response to the laboratory environment, with improvement in one or more life-history traits (Frankham & Loebel, 1992; Matos *et al.,* 2000b, 2002; Sgrò & Partridge, 2000; Gilligan & Frankham, 2003). However, discordances exist among studies with respect to specific patterns of adaptive response, particularly for traits with an uncertain connection to fitness in the laboratory environment (e.g. starvation resistance; see Matos *et al.,* 2000b, 2002; Hoffmann *et al.,* 2001). Disparities may also occur due to different methodologies. Most laboratory adaptation studies employ a comparative approach (e.g. Frankham & Loebel, 1992; Latter & Mulley, 1995; Hoffmann *et al.,* 2001; Woodworth *et al.,* 2002; Gilligan & Frankham, 2003; Griffiths *et al.,* 2005) while only a few analyze detailed real-time evolutionary trajectories (Matos *et al.,* 2000b, 2002, 2004; Krebs *et al.,* 2001). Comparative studies rely on the analysis of different laboratory populations founded from a particular natural site in different years to infer an evolutionary trajectory (e.g. Sgrò & Partridge, 2000; Hoffmann *et al.,* 2001; Gilligan & Frankham, 2003; see chapter 3). In this general type of experimental design, the most recently founded populations are assumed to reflect the initial fitness performance of long-established populations. Although potentially informative, this approach has several untested assumptions that may limit the validity of interpretations of the evolutionary patterns observed (see Leroi *et al.,* 1994b; Matos & Avelar, 2001; Matos *et al.,* 2004). These limitations become particularly important when studying characters with complex evolutionary patterns (Matos *et al.,* 2004).

The studies presented here advocate a quite different approach to the study of laboratory evolution. Instead of a comparative approach, the use of direct characterization of temporal changes within populations is employed to infer evolutionary dynamics. This is done through the analysis of the evolutionary trajectories in experimental populations as they evolve in novel laboratory environments, using long-established laboratory populations as controls.

The series of studies presented in this thesis are part of an ongoing research program aiming to characterize in detail the process of biological adaptation to a new environment. This research has particularly focused on the study of changes in life-history traits during laboratory adaptation, using *Drosophila subobscura* as model organism (Matos *et al.,* 2000b; 2002; 2004). This has been achieved by the analysis of

the evolutionary trajectories of mean values of these fitness related traits. The initial study obtained indications of laboratory adaptation through an improvement in fecundity and starvation resistance in a recently introduced population ("W") - founded from a collection of samples from a wild population of Sintra, Portugal - during the first 14 generations of laboratory culture (Matos *et al.,* 2000b). However, this study was limited by the lack of replicate populations. Therefore, additional work was performed to further investigate the dynamics of laboratory adaptation. With that purpose, a new experiment was conducted with extensive replication (5) and also longer established populations as reference, populations that were derived from the reference population used in Matos *et al.,* 2000b. This study revealed evolutionary convergence occurring as recently established populations ("NW" populations, founded from the same natural location) increased their performance for several life-history traits, thus approaching the values presented by the longer established populations ("NB" populations) - see figure 1, for a graphical representation of these laboratory populations. The observed improvement in several life-history traits suggests positive genetic correlations during the early stages of adaptation to a novel environment (Service & Rose, 1985). A decline in this correlation is expected as populations approach evolutionary equilibrium, leading to changes in the evolutionary trajectories of less relevant traits (Matos *et al.,* 2004).

The pattern and rate of convergence during the first 14 generations differed between the two studies mentioned, either due to changes in the founder (wild) population or in the laboratory environment between experiments (Matos *et al.,* 2002). Micro-evolutionary processes thus are apparently sensitive to genetic and environmental conditions. Nevertheless, the importance of the genetic component in the differences observed between studies could only be correctly addressed through the synchronous analysis – thus removing sources of environmental "noise" - of populations derived from different wild sources. Finally this body of data was also used to test the accuracy of the comparative method to infer evolutionary patterns. We found that correct evolutionary inferences could be generated from comparative data for fecundity related traits although the same did not apply to starvation resistance traits (Matos *et al.,* 2004).

The above-mentioned issues as well as other relevant aspects of evolutionary processes are pursued in this thesis. Specifically, I study patterns of short-term evolutionary response; repeatability of initial adaptation to the laboratory environment; the importance of effects of foundation arising from differences in genetic background and other contingent events in adaptive processes; long-term domestication patterns,

spanning up to 200 generations in the laboratory. Furthermore, the accuracy of the comparative method to generate precise evolutionary trajectories will be further tested using our new body of data. This work is summarized in the thesis plan below.

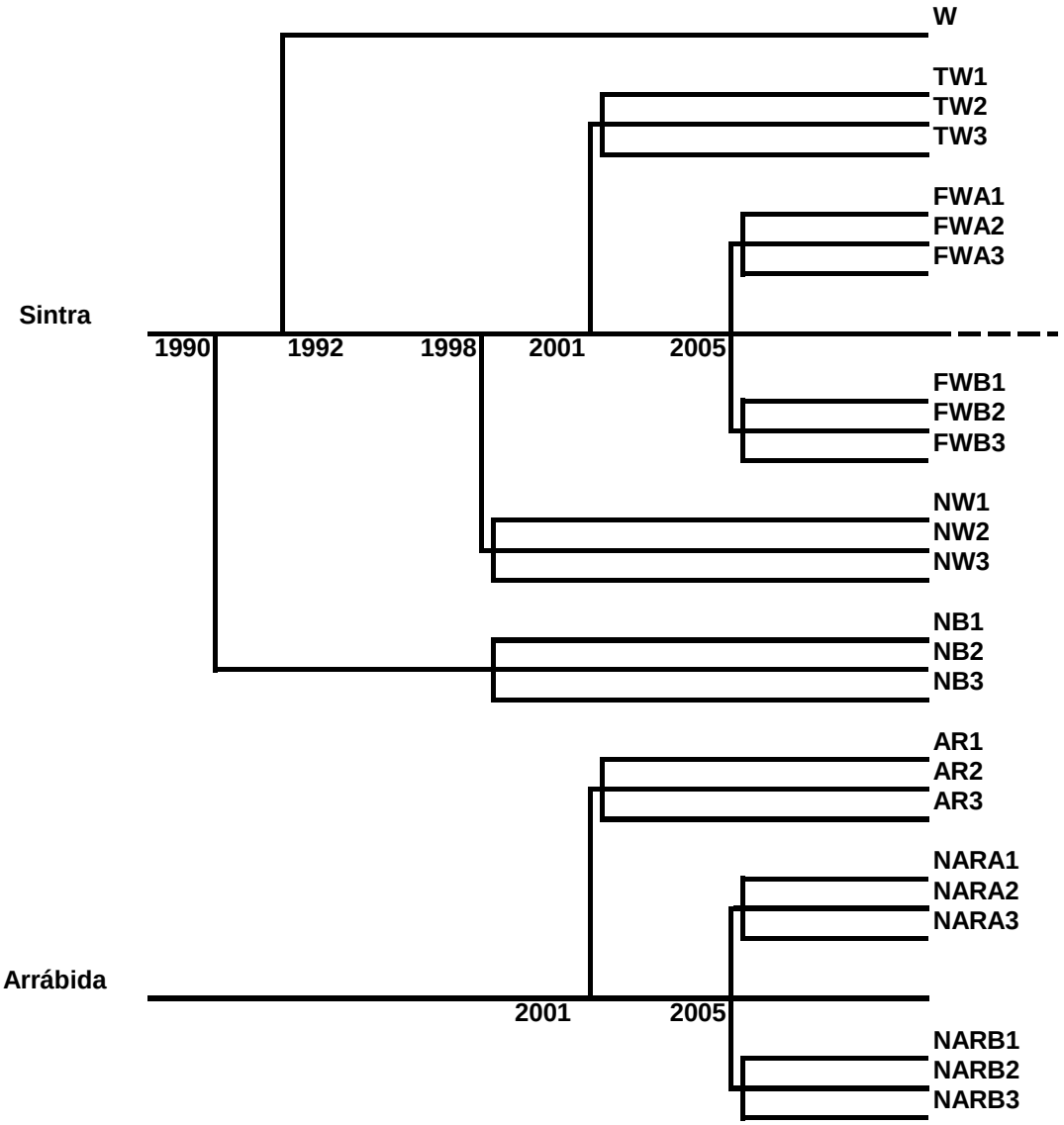

Figure 1. "Phylogeny" of all laboratory populations, indicating the original wild location and year of foundation. The NB populations are used as controls. When the 2005 foundations were performed the populations established in 1990 (NB), 1992 (W), 1998 (NW) and 2001 (AR and TW) were, respectively, at generation 181, 157, 91 and 45. The foundations of Arrábida and Sintra were carried out synchronously (in both 2001 and 2005). Collections in different years were done at the same location (pinewoods in Sintra and Arrábida).

1.3 Molecular genetic changes during laboratory adaptation

Alongside with the traditional approach of life-history evolution, molecular tools have been increasingly used to investigate evolutionary processes and their consequences in the genetic composition of natural populations. These molecular techniques have now been applied to several areas of biological research such as population genetics, phylogeography, and also conservation studies (Hartl & Clark, 1997; Li, 1997; Sunnucks, 2000; Frankham *et al.,* 2002; Avise, 2004; DeSalle & Amato, 2004).

Both fundamental evolutionary studies and conservation-oriented approaches have dealt predominantly with the study of genetic diversity. The main aim is to understand the patterns of genetic variability both within and among species and the causes of temporal changes in these patterns. The term genetic variability refers to the variety of different alleles and genotypes present in a population, and is ultimately reflected in variation between populations for relevant traits such as morphological, behavioral, and physiological traits (Frankham *et al.,* 2002). Since the 1990´s, the use of molecular markers has become a widespread approach to the characterization of the genetic properties of populations. These have included the assessment of different genetic measures such as allelic diversity, observed and expected heterozygosity or gene diversity, and allelic frequencies. The expected heterozygosity is one of the most widely used genetic parameters and gives the probability that two alleles chosen at random in a population are different (Nei, 1987). Comparisons of allelic frequencies among populations have also allowed the calculation of different genetic distances and differentiation measures, such as the F_{st} index (Wright, 1931, 1951). F_{st} can be defined as a standardized measure of among population differentiation, calculated as the ratio of variation among populations (V_b) to total population variation ($V_b + V_w$), as $V_b /(V_b + V_w)$, with V_w being the variation within populations.

Molecular markers have been used in population genetics studies under the assumption of neutrality, i.e. assuming that alleles are without effects on fitness. Under the neutral theory, allele frequencies in a population will change from generation to generation, due to the stochastic process of sampling a finite number of gametes each generation (i.e. genetic drift), with the magnitude of these changes being dependent on the effective size of the population (Crow & Kimura, 1970). The effective population size (N_e) is defined as the size of an idealized Wright-Fisher population (Fisher, 1930; Wright, 1931), which would give the same value of some specified genetic property as

in the population in question (Crow & Kimura, 1970). This N_e concept is central in population genetics, evolution, and conservation biology. It predicts the rate of decrease of heterozygosity or the rate of increase in homozygosity (i.e. inbreeding) through time – then being the inbreeding effective size - and measures the variance of change in allele frequency due to one generation of sampling – as the variance effective size (see Wang, 2005). In small N_e populations, genetic drift in allele frequencies will be higher and thus genetic polymorphism is expected to decrease due to allele fixation or loss.

Neutral molecular markers allow us to infer population breeding system and overall demographic structure. These include insights into past demographic history (e.g. occurrence of population bottlenecks) and the estimation of relevant parameters such as the effective population size (e.g. Nei & Tajima, 1981; Waples, 1989; Luikart *et al.,* 1999; Wang, 2001; see Wang, 2005, for a review of the different N_e estimation methods).

On the other hand, the genetic information obtained through molecular techniques has also been used to test for the effects of natural selection at the molecular level. Under this approach, the neutral theory has become the null hypothesis, instead of the presumed explanation for any observed patterns of molecular genetic variation (Hey, 1999; Fay *et al.,* 2002; Ford, 2002).

Several molecular genetic approaches have been developed to study the effects of selection on genetic variation at the molecular level (Lewontin & Krakauer, 1973; Beaumont & Nichols, 1996; Schlötterer, 2002a,b). This has been achieved by inferring natural selection through the direct analysis of changes in allele frequencies, DNA sequence variation, polymorphism data and microsatellite variability (see Ford, 2002; Schlötterer, 2002b for a review). In this context, it has been proposed that genome-wide scans can be a valuable tool to detect natural selection at the molecular level and ultimately to search for adaptive trait loci (Schlötterer, 2002a, 2003; see Storz, 2005 for a review). This would generate several candidate genes that should then be tested, with the ultimate goal of detecting the genetic differences that lead to phenotypic (and adaptive) differences.

One of these approaches aims to identify genes involved in local adaptation through the extensive screening of the genome using neutral molecular markers – *hitchhiking mapping* (Harr *et al.,* 2002; Schlötterer, 2003). It relies on the principle of *genetic hitchhiking* (Maynard Smith & Haigh, 1974), in which a selectively neutral region that is linked to a positively selected locus (or portion of the genome) will

decrease considerably in genetic variability as the locus (containing a beneficial mutation) becomes fixed, a so-called selective sweep. The strength of genetic hitchhiking events depends on several factors, such as the selection intensity, the frequency of beneficial mutations, and the recombination rate (Kaplan *et al.,* 1989; Wiehe & Stephan, 1993; Barton, 2000). To perform these multilocus screens, Schlotterer (2002b) developed a test statistic based on microsatellite variability ratios in a single population in order to detect loci that deviate from neutral expectations.

Microsatellite markers are particularly suited for this approach given that they are widely abundant across eukaryotic genomes, highly polymorphic (as a result of a high mutation rate relative to other loci), and relatively easy to score. These neutral markers are nuclear sequences of nucleotides made up of a single sequence motif (no more than six bases long) that is tandemly repeated (e.g. CACACACACA) – see Goldstein & Schlötterer (1999). These loci can reach a length of a few hundred base pairs (bp). Microsatellite polymorphisms derive from variability in length rather than in the primary sequence, being thus easily detected. These length changes are primarily due to replication slippage, as a result of a misalignment of replicating DNA strands (see Ellegren, 2004 for an overall review on microsatellites).

Assuming that most microsatellite markers are neutral, the screening of several microsatellite loci will allow us to disentangle between demographic effects, that will likely affect all loci, and the imprint of natural selection, expected to be more locus-specific. The microsatellite screening is then used to identify regions that differ from the remainder of the genome – the small portion of the genome subjected to recent hitchhiking events. Also, as this screening method uses ratios between populations to estimate changes in genetic diversity through time at each particular locus, it is also largely independent of microsatellite mutation rates and patterns (Schlötterer, 2002b). This procedure is thus likely to allow us to detect regions of the genome (and eventually specific genes) involved in local adaptation (Schlötterer, 2002a,b). Given the abundance of microsatellite sequences in the genome, these screens are expected to generate several candidate genes involved in adaptation. This approach has been most commonly used to search for adaptive mutations particularly in *Drosophila melanogaster* (e.g. Harr *et al.,* 2002; Kauer *et al.,* 2003) but has also been applied to other species, such as the house mouse (e.g. Ihle *et al.,* 2006) and humans (e.g. Payser *et al.,* 2002).

Several studies have also combined both neutral genetic and quantitative phenotypic traits to address possible causes of population differentiation (e.g. Koskinen

et al., 2001; Storz, 2002). These studies rely particularly on molecular markers (e.g. microsatellite loci) to ascertain the genetic divergence between populations due to genetic drift, assuming neutrality of these markers (the neutral null hypothesis). On the other hand, the quantitative divergence commonly found for morphological or life-history traits may be due to genetic drift and/or natural selection, leading to different expectations according to the specific evolutionary mechanisms involved. As a result, through the comparison of quantitative ("Q_{st}") and neutral genetic ("F_{st}") measures of population divergence, several hypothetical scenarios can be tested (see Merilä & Crnokrak, 2001; McKay & Latta, 2002). If F_{st} equals Q_{st} then quantitative differentiation could have been caused by genetic drift alone. On the other hand, if Q_{st} exceeds F_{st} directional natural selection is arguably the main factor causing population divergence. Finally, F_{st} higher than Q_{st} results are interpreted as evidence of stabilizing selection favoring the same phenotypes in the different populations studied.

Despite their usefulness, molecular genetic approaches have seldom been applied to study adaptive processes in laboratory populations (but see Porcher *et al.,* 2004; Morgan *et al.,* 2005). For instance, the use of laboratory populations maintained in carefully controlled conditions and subjected to well-defined selective regimes offers ideal conditions with which to test the assumptions of the above mentioned Q_{st} *vs*. F_{st} test. In one such study, Morgan *et al.* (2005) used laboratory populations of house mice directly selected for wheel-running activity to empirically evaluate Q_{st} *vs*. F_{st} comparisons. The authors concluded that Q_{st} *vs.* F_{st} comparisons generally produce correct evolutionary inferences. Porcher *et al.* (2004) have also addressed this issue by testing for a causal relationship between selection heterogeneity and quantitative differentiation (Q_{st} values) in greenhouse metapopulations of *Arabidopsis thaliana*. The authors demonstrated that the effects of selection heterogeneity (using varied selection differentials) were clearly detectable in the Q_{st} estimates, supporting the relevance of this parameter as an estimator of genetic differentiation in quantitative traits.

Laboratory studies using molecular approaches have been primarily focused on conservation biology issues, such as the development of strategies to limit genetic variability decline due to genetic drift, and the study of the impact of effective population size on rates of genetic variability decline among captive populations (e.g. Montgomery *et al.,* 2000; England *et al.,* 2003; Gilligan *et al.,* 2005; Toro & Caballero, 2005; Rodríguez-Ramilo *et al.,* 2006). In addition to its practical relevance, these conservation studies have also raised critical questions concerning the analysis of

molecular variation and its connection with adaptive evolution (Hedrick, 1999; Crandall *et al.,* 2000; Reed & Frankham, 2001). There has been controversy in the literature with regard to the possible association between genetic variation in quantitative traits and molecular markers (e.g. Soulé & Zegers, 1996 cf. Butlin & Tregenza, 1998). A meta-analysis study demonstrated that measures of molecular and quantitative variation show a low correlation (Reed & Frankham, 2001). Several factors make this outcome understandable. First of all, quantitative traits may be affected by non-additive gene action and genotype-by-environment interactions leading to non-intuitive complexities. A higher mutational input in quantitative trait loci (due to the high number of loci potentially involved) relative to molecular markers can also lead to disparate amounts of genetic variation. Furthermore, many quantitative traits are expected to be under selection. In particular, directional selection will most likely lead to depletion of standing genetic variance to a higher degree relative to neutral molecular markers. On the other hand, balancing selection may favour the maintenance of genetic variance for the selected traits, rendering accurate predictions or associations between measures of molecular and quantitative variation even more difficult.

Finally, statistical power differs between quantitative genetic and molecular measures (Reed & Frankham, 2001). This implies that low (or high) levels of molecular variation do not necessarily reflect low (or high) quantitative-genetic variation and therefore evolutionary potential. Despite the above, Reed & Frankham (2003) have found evidence of a correlation between fitness and overall measures of genetic diversity. The authors argue that the positive correlation found between molecular heterozygosity and fitness suggests that heterozygosity can be an indicator of population fitness, through its association with population size. This is explained by the fact that drift and inbreeding depression as a result of low effective population sizes will likely lead to both lower molecular variation and also reduced fitness. However, despite the significant correlation obtained, the determinants and covariates of fitness used in this study – heritabilities, heterozygosity, and population size – explained only 15-20% of the variation in fitness. Thus the question remains, whether an association between genetic diversity in molecular markers and the potential to adapt in face of environmental changes is legitimate (Hedrick, 1999; Reed & Frankham, 2001; cf. Reed & Frankham, 2003).

Given the contribution that molecular population genetics can provide to the study of adaptation, the molecular changes during laboratory evolution will be studied in this thesis, as a complement to the analyses of phenotypic evolution. This approach allows a detailed genetic characterization of laboratory populations in terms of genetic variability (and its rates of decline through time), effective populations sizes during laboratory evolution, genetic differentiation between and within populations through time, etc…. Furthermore, the analysis of possible changes in microsatellite allele frequencies and genetic variability through time in controlled laboratory populations is also a potentially valuable approach to the search for genes involved in adaptive processes. The impact of positive selection in our molecular data, as a result of a possible hitchhiking event, will be tested using temporal samples from our laboratory populations, differing in the number of generations in the laboratory. Finally, molecular and quantitative data will be compared to search for possible associations between molecular genetic variability and evolutionary potential and to test the validity of evolutionary inferences based on comparisons of quantitative and molecular genetic differentiation (see details below).

1.4 Thesis Plan

This chapter provided a general introduction to the experimental evolution field, the setting of this thesis. The general principles of experimental evolution studies were here outlined. Emphasis was placed on recent laboratory studies of adaptation, providing a critical review of the major conclusions they have reached. The remaining five chapters deal with the original contribution of this thesis to the body of empirical studies that use laboratory evolution to characterize the evolutionary patterns and processes underlying local adaptation.

In **Chapter 2**, short-term evolutionary responses and the impact of genetic background effects on adaptation to the laboratory environment are analyzed through the study of two sets of populations derived from different natural locations ("AR" populations from Arrábida and "TW" populations from Sintra, Portugal) in 2001 – see figure 1. The synchronous analysis of these populations will reduce the confounding effects of environmental factors during laboratory evolution and allow testing of the specific impact of different genetic backgrounds in the adaptive patterns during the first 40 generations of laboratory evolution.

Long-term domestication is characterized through the detailed study of evolutionary trajectories of two different sets of populations – "NW" and "NB" populations (covering the first 86 generations of laboratory evolution of the former and generations 94-176 of the latter). These represent the longest studies of evolutionary domestication in a sexual diploid organism allowing direct inference of evolutionary trajectories, i.e. real-time evolution.

Chapter 3 summarizes the laboratory adaptation data obtained for NB, NW, AR and TW populations (extended for 8 additional generations relative to chapter 2) and presents a general revision of the literature on studies of domestication in *Drosophila*. The impact of different evolutionary forces and processes (e.g. natural selection, genetic drift, inbreeding…) on the patterns observed is discussed. In order to test the accuracy of the comparative method in predicting real evolutionary trajectories an additional analysis is also performed, including data from a more recent foundation (done in 2005). This is done using the information obtained in a single, synchronous phenotypic assay of all the different laboratory populations derived from independent foundations (from the same natural site) to infer evolutionary trajectories. These trajectories are compared to the real-time evolutionary trajectories obtained by the analysis of the temporal changes within populations through time. A final discussion contrasts results across studies, both by our own team and by others.

In **Chapter 4** the repeatability of evolutionary processes is tested, using the growing body of data obtained from several collections differing either in space (Sintra and Arrábida) or time (across 3 different studies started in 1998, 2001 and 2005). Relying on data collected from independent foundations in these two different locations across years (2001 and 2005), both geographical (i.e. location of sampling) and temporal (i.e. year of sampling) effects on the genetic composition of populations can be tested as potential sources of contingency in the adaptive process. Finally, this study also tests the general relevance of sampling genetic effects (by performing independent collections within each location) as sources of variation in an adaptive process, as opposite to effects of foundation derived from distinct geographical origin. This is achieved by performing two independent collections (in 2005) at each one of the two natural locations (see figure 1).

In **Chapter 5** an extensive genetic characterization of the "NW" populations (in their 49^{th} generation of lab culture) is presented, through the analysis of 10 microsatellite loci. This study focuses on NW genetic variability levels, genetic

differentiation and decline in genetic variability through time. A comparison of the NW molecular genetic composition with that of "TW" populations (three replicates) in their 3rd generation is performed, using the TW populations as a proxy for inferring the initial genetic variability upon laboratory foundation. Different approaches for the estimation of effective population size using the temporal method are also addressed. The levels of genetic variability and differentiation between populations under prolonged captivity are discussed and compared to those found in other studies.

Chapter 6 presents a detailed study of the temporal dynamics of microsatellite allele frequency changes in two sets of populations with distinct genetic backgrounds ("AR" and "TW" populations). In this particular work, the microsatellite and life-history data obtained for these two sets of populations during their first 40 generations of laboratory culture are compared. Important descriptive parameters such as initial genetic variability, rate of variability decline and effective population sizes are measured and compared between the two sets of populations evolving in synchrony. A test statistic, described in Kauer *et al.* (2003), using heterozygosity ratios is also applied to test for positive selection in our microsatellite dataset. The magnitude of temporal genetic changes in molecular markers (F_{st}) and quantitative traits (Q_{st}) during laboratory adaptation are also compared and discussed.

In **Chapter 7** a general discussion of the relevant findings of this thesis is provided, combining all the new information and highlighting its contribution to the understanding of the evolutionary dynamics of life-history traits and molecular markers during laboratory adaptation. This thesis ends with a final consideration of promising research areas within experimental evolution and the new techniques available to such evolutionary studies.

I will now briefly highlight the main questions addressed in this thesis, throughout the several chapters presented above.

1.5 General Questions addressed:

Do all life-history traits improve during laboratory culture? Given the relevance of life-history traits (adult and juvenile) to overall fitness, an improvement in these traits is usually expected as a result of adaptation to a new environment. The evolutionary response observed will depend on diverse aspects such as the amount of additive genetic

variability, genetic constraints (e.g. trade-offs), and the relative importance of each life-history trait for functioning in the new laboratory environment. Long-term laboratory evolution might also lead to a decline in the performance of less relevant traits as a result of relaxed selection and the accumulation of deleterious alleles (chapter 2 and 3).

Does this rate of improvement decline with time? Following an initial evolutionary response, a decline in the rate of improvement of each life-history trait is expected as the performance of the adapting populations approaches the evolutionary optimum in the new environment. In this context, the observation of a *plateau* as a result of the complete cessation of improvement is possible, although its timing is rather unpredictable depending on the genetic basis of each trait and the strength of selection (chapter 2 and 3).

Is there repeatability during laboratory evolution? Populations differing in their genetic background are likely to show disparate evolutionary patterns in the laboratory due to different additive genetic variances, I_{gxe}, stochastic events of population history, etc. These factors can influence different aspects of the evolutionary response such as the rate of improvement in an early stage of adaptation and also the timing of a possible slowing down of adaptation (or even *plateau*). Do these factors hinder general repeatability of evolutionary patterns? Or does natural selection override these initial differences, promoting uniformity in evolutionary responses? The repeatability of the evolutionary patterns of life-history traits will be tested by combining information from several independent foundations differing either in time and space (collections from two different natural locations). The importance of contingent factors (e.g. prior evolutionary history; chance events) in adaptive evolution can thus be addressed. The importance of effects of foundation (as a result of geographical origin and/or sampling effects within the same region) in adaptation processes will also be tested (chapter 2 and 4).

Do comparative analyses accurately reflect the real evolutionary trajectories of populations? Despite the usefulness of the comparative method to infer evolutionary processes, its ability to provide detailed information about adaptive dynamics is questionable (Leroi *et al.,* 1994b; Matos *et al.,* 2004). In this work, new data will be used to test the accuracy of inferences of evolutionary trajectories using a comparative

method (involving a synchronous assay of several populations with a different number of generations in the laboratory), compared with real-time evolutionary trajectories (chapter 3).

Do laboratory populations retain considerable genetic variability? Given their confinement, laboratory populations are expected to lose genetic variability due to drift. However, few studies have tried to quantify this genetic loss empirically across dozens of generations in moderately sized populations (with N≈1000) (chapter 5 and 6).

Is the rate of decline in genetic variability similar in different laboratory populations? The rate of genetic variability decline will depend mainly on the effective population size via its direct impact in the magnitude of genetic drift effects acting on these populations. On the other hand, the effective population size in laboratory populations under selection is likely to be affected by evolutionary history and thus could potentially vary between laboratory populations and even in the same population during the course of laboratory culture (chapters 5 and 6).

Does molecular variation reflect evolutionary potential? Recent studies have shown a weak correlation between molecular and quantitative variation (e.g. Reed & Frankham, 2001). Thus overall molecular variation is likely to be only a pale reflection of the additive genetic variation – the fuel for natural selection. However, other studies have suggested an association between different measures of genetic variation (including molecular) and the evolutionary potential of populations (e.g. England *et al.*, 2003; Reed & Frankham, 2003). The combined analysis of life-history and molecular genetic (microsatellite loci) data of different populations during adaptation to a new environment will hopefully allow us to clarify some of these issues (chapter 6).

Can directional natural selection be inferred through Q_{st} *vs.* F_{st} comparisons? This has been a much used approach in recent evolutionary biology studies (e.g. Merilä & Crnokrak, 2001; McKay & Latta, 2002). The combination of both quantitative and molecular genetic data from our evolving laboratory populations will allow us to test the consistency of this approach given the *a priori* expectation of an important role of directional selection in laboratory evolution – $Q_{st} > F_{st}$ (chapter 6).

Can natural selection in the laboratory be traced through molecular data? It should be possible to detect the impact of natural selection on molecular variation as a result of hitchhiking when there is linkage disequilibrium between a neutral site and a positively selected region (Maynard Smith & Haigh, 1974). Experimental evolution studies, through the implementation of defined selective regimes and the ability to follow the genetic changes in the evolving population, can be a powerful approach to detect natural selection also at the molecular level (chapter 6).

These are some of the general questions that I aim to address here, questions that in general stem from the study of adaptive processes. The detailed study of a real-time adaptive process in outbred populations colonizing a new environment is a robust approach to tackle these questions. Here I present a series of laboratory adaptation studies in populations of *Drosophila subobscura* in which both phenotypic traits and genetic markers are analyzed in a temporal perspective.

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Chapter 2.

Evolutionary domestication in *Drosophila subobscura*

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Evolutionary domestication in *Drosophila subobscura*

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inbreeding;
laboratory adaptation.

Abstract

The domestication of plants and animals is historically one of the most important topics in evolutionary biology. The evolutionary genetic changes arising from human cultivation are complex because of the effects of such varied processes as continuing natural selection, artificial selection, deliberate inbreeding, genetic drift and hybridization of different lineages. Despite the interest of domestication as an evolutionary process, few studies of multicellular sexual species have approached this topic using well-replicated experiments. Here we present a comprehensive study in which replicated evolutionary trajectories from several *Drosophila subobscura* populations provide a detailed view of the evolutionary dynamics of domestication in an outbreeding animal species. Our results show a clear evolutionary response in fecundity traits, but no clear pattern for adult starvation resistance and juvenile traits such as development time and viability. These results supply new perspectives on the confounding of adaptation with other evolutionary mechanisms in the process of domestication.

Introduction

The domestication of plants and animals is historically one of the most important topics in evolutionary biology, figuring prominently in Darwin's *Origin of Species*. Traditionally, the term 'domestication' refers to the genetic changes undergone by our commensal species, from dogs to agricultural animals to grains to legumes, sometimes with an additional connotation related to behavioural change, especially reduction in 'wildness' (Soanes, 2003). A more useful definition, however, for scientific purposes is that *domestication is the evolutionary genetic change arising from the transition of a population from nature to deliberate human cultivation*. In some laboratory populations, such as those of *Drosophila* or *Escherichia coli*, the 'state of nature' may be laboratory culture under a sequence of ill-defined, arbitrarily or haphazardly changing conditions (cf. Matos *et al.*, 2002; Lenski, 2004).

Domestication, as defined here, is of both practical and theoretical scientific interest. One of the enduring problems in the breeding of both plants and animals is the interpretation of the evolutionary conditions that they have been subjected to, a topic that was a particular favourite of Darwin (1859, 1883). The development of modern animal and plant breeding has depended, in part, on the spread of this type of evolutionary understanding from theorists, like Darwin, to practical breeding. Understanding the impact of captivity is also becoming prominent in conservation genetics, as more and more species are being maintained in *ex situ* conservation programmes (Frankham *et al.*, 2002).

For evolutionary biology itself, domestication provides one of the more important contexts for experimental evolution. It is both a background to evolutionary studies of diversification under selection (e.g. Rose *et al.*, 2004) and an important topic in itself. In studying domestication in well-defined laboratory experiments, we can measure in detail the evolutionary process with replication and specific environmental controls. In this context, such key evolutionary processes as adaptation and inbreeding occur transparently and reproducibly, a fruitful setting for testing biological hypotheses (see Mueller & Joshi, 2000; Houle & Rowe, 2003; Prasad & Joshi, 2003).

Domestication of *Drosophila* populations that have been founded from wild samples has been studied using two different approaches. First, comparison of populations that have and have not been subject to particular domestication regimes (e.g. Sgrò & Partridge, 2000;

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Hoffmann *et al.*, 2001; Krebs *et al.*, 2001; Gilligan & Frankham, 2003; Griffiths *et al.*, 2005). Secondly, temporal analysis of the evolutionary trajectories of domesticated populations since their foundation from the wild (e.g. Matos *et al.*, 2000b, 2002), our approach for the last 15 years.

In particular, we have been studying evolutionary convergence between the recent and the long-established populations, suggesting laboratory adaptation, particularly in fecundity traits (Matos *et al.*, 2000b, 2002). In the present study, we extend these previous studies to include more generations, more fitness-related traits, and two new, independent, synchronous foundations. Here we offer the most detailed view yet of domestication in an outbreeding animal species, with new information on the confounding of adaptation, founder effects and inbreeding in the process of domestication.

In this study these specific questions were addressed:

Are there directional patterns of adaptation across traits?
Are there plateaus in long-term domestication?
Do long-maintained populations show progressive inbreeding depression?
Is there a temporal increase in divergence between replicate populations?
How important are effects of foundation for evolutionary patterns and processes during local adaptation?

Finally, we address the relevance of these laboratory studies to practical domestication and conservation issues, such as *ex situ* breeding programmes.

Materials and methods

Foundation and maintenance of the laboratory populations

Four sets of wild-caught samples of *Drosophila subobscura* were obtained (Fig. 1). In 1990, the 'B' population was founded from collections in a pinewood near Sintra, Portugal (Matos *et al.*, 2000b). In 1998, another population, 'NW', was founded, also from Sintra collections, by which time the B population was in its 90th generation. Two generations later, B and NW were split into five replicate populations, referred to as NB_{1-5} and NW_{1-5} respectively (Matos *et al.*, 2002).

In 2001, two additional foundations were carried out, one from Sintra, called 'TW' here, and another from a new location, Arrábida (about 50 km from Sintra), called 'AR' here. The TW population was founded from 110 female and 44 male insects and the AR population from 59 female and 24 male insects. After two generations in the laboratory they were both split into three replicate populations, TW_{1-3} and AR_{1-3}.

From the moment the populations were brought into the laboratory, they were all maintained under the same conditions: discrete generations of 28 days, reproduction close to the time of peak fecundity, a controlled temperature of 18 °C, and controlled densities. Population sizes were usually between 600 and 1200 individuals (Matos *et al.*, 2000b, 2002).

Life-history trait assays

Assays of adult traits

Assayed flies were transferred daily as mated pairs to laying vials containing freshly prepared medium. The total number of eggs laid per female insect was counted every day for the first 12 days, after which starvation resistance was assayed. Five characters were analysed: age of first reproduction (number of days between emergence and the first egg laying – 'A1R'), early fecundity (total number of eggs laid during the first week – 'F1–7'), peak fecundity (total number of eggs laid between days 8 and 12 – 'F8–12'), female and male starvation resistance (number of hours until death, registered every 6 h after transfer to a vial with plain agar – 'RF' and 'RM' respectively).

For the NW populations, assays were carried out during their generations 4, 8, 13, 15, 33, 43, 47, 50, 52, 53, 58, 60, 64, 66, 71, 78 and 86, with corresponding assays of NB populations that had already been

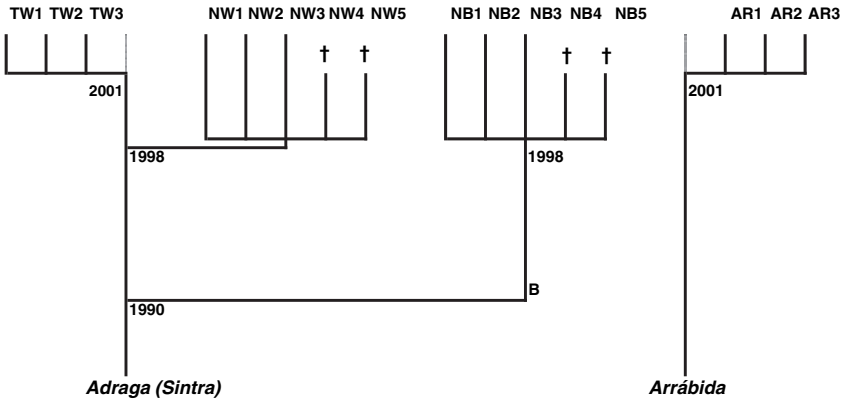

Fig. 1 Phylogeny of the laboratory populations; each main line starting at the natural location (Sintra and Arrábida) represents the natural population where collections were made; three collections were done in Sintra and one in Arrábida, the years being marked in the derived branches representing the foundation of the laboratory populations.

maintained for that number of generations plus 90. Sample sizes per replicate population were between 14 and 21 pairs (Matos *et al.*, 2002). Because of the accidental loss of two replicates of both NW and NB at generation 50 of the former, only the data of replicates NW_1 to NW_3 and NB_1 to NB_3 will be used in all analyses.

For the AR and TW populations, assays were carried out during their generations 3, 4, 6, 7, 12, 14, 18, 20, 25, 32 and 40, with sample sizes between 14 and 18 pairs per replicate population. All assays of AR and TW flies involved simultaneous assays of NB and NW populations (with an additional 136 and 46 generations after introduction in the laboratory respectively), except for the assay at generation 3, when the NW populations were not assayed.

Assays of juvenile traits

For each assay a collection of about 70 eggs per vial was made using eggs laid over a period of 4–6 h. Sample sizes were usually eight vials per replicate population. Development time for female (FDT) and male (MDT) insects was estimated as the number of hours from egg to emergence of imagos.

Juvenile viabilities (VIAB) were estimated for each vial as the total number of adults collected per vial divided by 70. Assays for juvenile traits of NW populations were done at generations 3, 4, 6, 11, 20, 48, 51, 54, 59, 65, 73 and 81. NB populations were assayed in parallel at the corresponding generations (i.e. at generation 93, 94, 96 etc.). AR and TW populations were assayed at their generations 5, 8, 13, 19, 27 and 35.

Statistical methods

All data analysis was performed using STATISTICA and EXCEL. All regressions were Type I least-squares linear regressions (Sokal & Rohlf, 1995). The regression analysis was carried out using the mean values of traits for each replicate population as the dependent variable and generation number as the independent variable. The analyses used *both* actual values for each population and the paired differences from NB populations between same arbitrarily numbered replicates for the evolutionary trajectories of NW, AR and TW populations. The analysis of paired differences relative to the longer established NB populations was used to minimize environmental noise that might reduce statistical power to detect evolutionary trends (Matos *et al.*, 2002). In all cases, the significance of the linear trajectory was determined by *t*-test using the average slope of the replicate populations, with the variation of these slopes among replicate populations as the sample variation.

In addition to straightforward analysis of the effect of domestication on individual characters as a function of the number of generations of domestication, we analysed the dependence of *evolutionary rate* on *early differentiation*. The *evolutionary rate* (slope of evolutionary trajectories during the first 14–15 generations) was estimated using, in each generation, the difference in character values between experimental populations and control populations divided by the latter. To characterize *early differentiation*, we averaged the character values for several assays centred around generation 6. This standardization ensured that scale effects did not bias the dependence of evolutionary rate on early differentiation. We then estimated the best linear model relating evolutionary rate to early differentiation and tested it using *t*-tests and ANCOVA *F*-tests.

Results

Long-term domestication: NW and NB populations

Adult traits

Early fecundity (days 1–7). We tested for a directional change in NB early fecundity data from generations 94 to 176 using a *t*-test. At a confidence value of $P < 0.05$, there was no significant deviation from zero, suggesting an absence of directional, evolutionary change among the NB populations during this period (see Table 1). The NW data over generations 4 to 86 showed a significant increase in early fecundity (see Table 1). The differences between NW and the reference NB populations are shown in Fig. 2, for NW generations 4 to 86. The analysis showed no significant directional trend for the difference between NW and NB with respect to early fecundity (see Table 1 and Fig. 2).

Peak fecundity (days 8–12). At a confidence value of $P < 0.05$, no directional change was obtained for NB populations (see Table 1). The analysis of NW populations showed no directional trend in NW peak fecundity using actual values, but a highly significant increase in the difference between NW and NB populations (see Table 1 and Fig. 3).

Age of first reproduction. For all NW and NB populations, there was no suggestion of a significant evolutionary trend for the trait, analysed as absolute values or as differences between NW and NB replicates (see Table 1).

Starvation resistance. The NB data showed no significant linear trend for female starvation resistance (see Table 1). NW female starvation resistance showed a significant decline, both in absolute terms and in comparison with NB. NW and NB male starvation resistance showed no significant evolutionary trend (see Table 1).

Tests for temporal stabilization and population divergence of adult traits. NB populations showed no significant directional trend over generations 94 to 176, in contrast to the consistent changes for fecundity traits in the NW populations assayed synchronously for their generations 4 to 86 (see Table 1). This suggests that a domestication plateau had already been reached in the NB populations, whereas an adaptive response to domestication occurred in the NW populations assayed at the same time, but over earlier generations of domestication.

Table 1 Slopes of least squares linear regressions of the several traits for each NB and NW replicate population. The analysis of each set of populations used the individual slopes as data points in a *t*-test; at the bottom line for each set of populations the average slope of the linear model is presented.

	Adult traits					Juvenile traits		
	NB populations, generations 94–176					NB populations, generations 93–171		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
NB1	−0.0083	0.1855	−0.1685	−0.0277	−0.0424	0.4038	0.2820	0.0016
NB2	0.0046	−0.1664	−0.3392	−0.0090	−0.0803	0.0226	−0.0346	0.0048
NB3	−0.0042	0.0721	−0.0951	0.0090	−0.0299	−0.1833	−0.0084	0.0038
Average slope	−0.0026 n.s	0.0304 n.s.	−0.2010 n.s.	0.0090 n.s.	−0.0509 m.s.	0.0810 n.s.	0.0797 n.s.	0.0034 m.s.
	NW populations, generations 4–86					NW populations, generations 3–81		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
NW1	−0.0157	0.6254	0.3791	−0.0554	0.1235	0.7072	0.6596	0.0030
NW2	−0.0021	0.4169	0.1655	−0.0433	−0.0055	0.2286	0.1510	0.0038
NW3	0.0024	0.2774	0.4039	−0.0355	−0.0252	−0.6593	−0.6903	0.0052
Average slope	−0.0051 n.s.	0.4399*	0.3162 m.s.	−0.0447*	0.0309 n.s.	0.0922 n.s.	0.0401 n.s	0.0040*
	NW-NB, generations 4–86 (of NW)					NW-NB, generations 3–81 (of NW)		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
NW1-NB1	−0.0074	0.4399	0.5476	−0.0277	0.1659	0.3034	0.3776	0.0014
NW2-NB2	−0.0067	0.5833	0.5047	−0.0344	0.0748	0.2060	0.1856	−0.0010
NW3-NB3	0.0066	0.2053	0.4991	−0.0446	0.0047	−0.4761	−0.6819	0.0013
Average slope	−0.0025 n.s.	0.4095 m.s.	0.5171***	−0.0356*	0.0818 n.s.	0.0111 n.s.	−0.0396 n.s.	0.0006 n.s.

Adult traits: age of first reproduction (A1R); early fecundity (F1–7); peak fecundity (F8–12); female starvation resistance (RF); male starvation resistance (RM). Juvenile Traits: female and male development time (FDT and MDT); viability (VIAB).
n.s., $P > 0.1$; m.s, $0.05 < P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

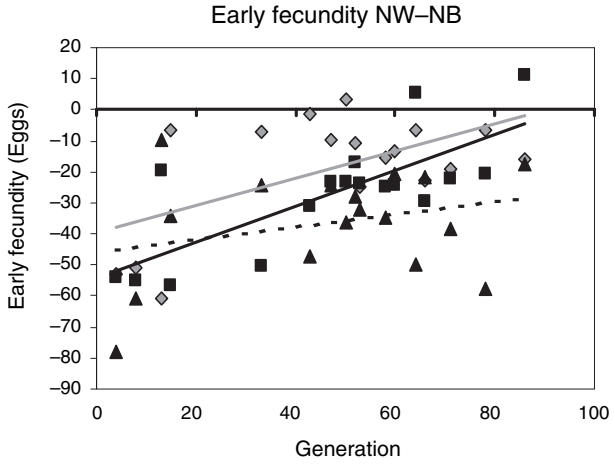

Fig. 2 Early Fecundity NW minus NB, NW Generations 4 to 86. Gray, diamonds, full line – first replicate population; Black, squares, full line – second replicate population; Black, triangles, dashed line – third replicate population.

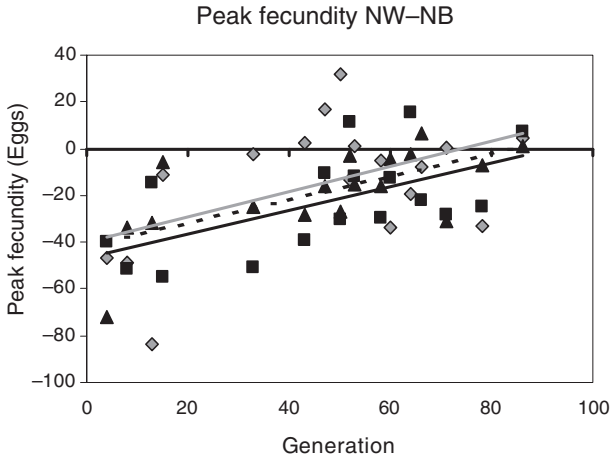

Fig. 3 Peak Fecundity NW minus NB, NW Generations 4 to 86. Gray, diamonds, full line – first replicate population; Black, squares, full line – second replicate population; Black, triangles, dashed line – third replicate population.

It is worth noting that log-linear models applied to the NW data usually did not show statistically improved fit over a linear model, which would be expected if evolutionary rates did decrease clearly with time. The exception was early fecundity, for which statistical analysis of log-linear models indicated a significant increase for NW populations relative to NB populations ($t = 6.465$, d.f. $= 2$, $P < 0.05$). This contrasts with the nonsignificant result obtained when the linear model was applied ($P = 0.066$). This result suggests a decrease in the rate of evolutionary change of early fecundity in the NW populations.

We failed to detect a consistent increase in the amount of divergence (measured by the coefficient of variability) among replicates over the course of domestication (data not shown). The only statistically significant result was a

decrease in divergence for peak fecundity among the NW replicates ($t = -2.21$; d.f. = 15; $P < 0.05$).

Juvenile traits

Development time. We found no significant longitudinal trends for this character in the NB and NW populations, for both males and females (see Table 1).

Viability. The NB viability data did not show any significant directional pattern (see Table 1). NW viability showed a significant upward trend, but there was no significant change in differences between NW and NB populations (see Table 1).

Test for replicate population diversification of juvenile traits. Data analysis of NW and NB populations showed no clear temporal changes in variability among replicate populations for all juvenile traits (data not shown).

Effects of wild source on domestication: TW vs. AR populations

Adult traits

Early fecundity (days 1–7). Figure 4 shows the temporal changes in TW and AR early fecundity, relative to NB values, during their first 40 generations of domestication. Both absolute values and differences relative to NB populations indicated a significant increase in fecundity for both AR and TW (see Table 2). A comparison of TW and AR populations did not indicate a significant difference in evolutionary rates between them (data not shown).

Peak fecundity (days 8–12). Figure 5 presents TW and AR peak fecundity as differences from NB values. The AR results were not significant, but the TW fecundities significantly increased relative to those of the NB populations. Dropping the use of the NB populations as a standardizing control, both TW and AR populations show a significant directional increase for fecundity (see Table 2). TW and AR did not differ significantly in the rate of temporal change for this trait.

Age of first reproduction. Unlike the NW populations, TW populations showed a significant improvement in this trait both for absolute values and relative to NB populations. For AR populations, no significant improvement was observed (see Table 2). TW and AR did not differ significantly in the rate of temporal change for this trait.

Starvation resistance. Starvation resistance shows a general lack of significant directional change, except for a significant increase in female starvation resistance among TW populations relative to NB. No significant difference was observed between the evolutionary rate of AR and TW populations for these traits.

Juvenile traits

Development time and viability. There were no significant directional trends or differences in evolutionary rates (see Table 2).

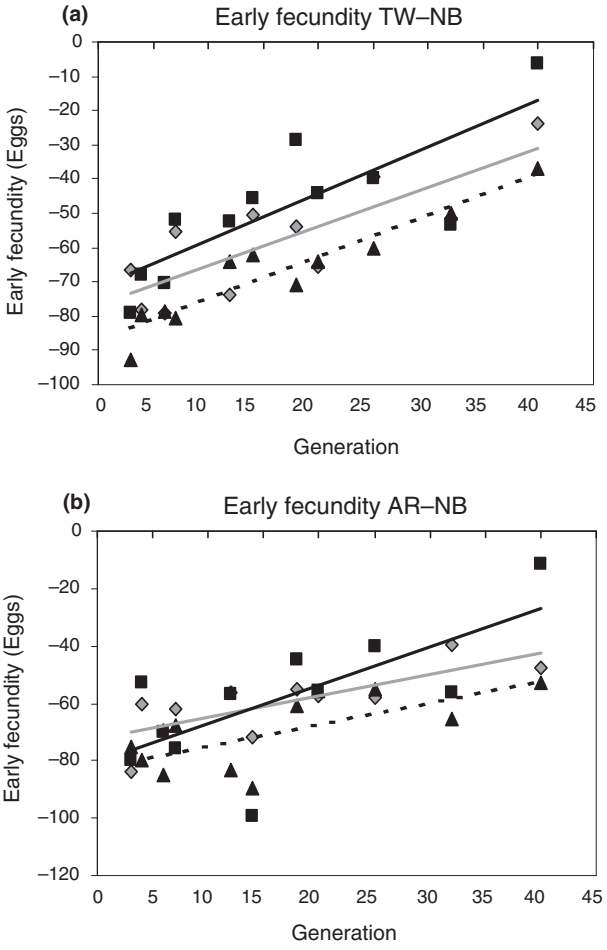

Fig. 4 Early fecundity evolutionary trajectories for TW minus NB and AR minus NB. (a) Early fecundity TW minus NB, Generations 3–40; (b) Early fecundity AR minus NB, Generations 3–40. Gray, diamonds, full line – first replicate population (AR or TW); Black, squares, full line – second replicate population (AR or TW); Black, triangles, dashed line – third replicate population (AR or TW).

Dependence of evolutionary rate on early differentiation

To analyse whether there is a dependence of evolutionary rate on early differentiation we estimated the linear regression of evolutionary rate on initial character value, as described in the *Materials and methods*, using data from simultaneous NB assays to standardize the results over repeated longitudinal assays. Figure 6 presents the results for the NW, TW and AR populations.

The results of ANCOVA and *t*-tests showed clearly that the replicated foundations differ significantly, with the exception of the NW vs. TW comparison ($P = 0.647$, for ANCOVA *F*-test with all replicate data points included; $P = 0.562$, for *t*-test).

In particular, it is worth noting the highly significant difference between AR and TW ($P = 0.000$, for ANCOVA

Table 2 Slopes of least squares linear regressions of the several traits for each TW and AR replicate population. The analysis of each set of populations used the individual slopes as data points in a *t*-test; at the bottom line for each set of populations the average slope of the linear model is presented.

	Adult traits					Juvenile traits		
	TW populations, generations 3–40					TW populations, generations 5–35		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
TW1	−0.0486	1.9221	2.0685	−0.0333	0.0514	1.1052	0.7637	−0.0003
TW2	−0.0680	2.0067	1.8781	−0.1584	0.0321	0.6164	0.2850	0.0049
TW3	−0.0577	1.4913	1.8718	−0.0396	−0.0627	0.3798	0.2209	0.0004
Average slope	−0.0581**	1.8067**	1.9395**	−0.0771 n.s.	0.007 n.s.	0.7005 m. s.	0.4232 n.s.	0.0017 n.s.
	TW-NB, generations 3–40 (of TW)					TW-NB, generations 5–35 (of TW)		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
TW1-NB1	−0.0387	1.1452	1.1799	0.0787	0.1722	−0.2790	−0.2111	−0.0039
TW2-NB2	−0.0668	1.3540	0.7198	0.0818	0.1693	−1.3616	−1.2568	0.0034
TW3-NB3	−0.0462	1.2177	0.9566	0.0365	0.0256	−1.9679	−1.5102	−0.0071
Average slope	−0.0506*	1.2390**	0.9521*	0.0657*	0.1224 n.s.	−1.2028 n.s.	−0.9927 n.s.	−0.0025 n.s.
	AR populations, generations 3–40					AR populations, generations 5–35		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
AR1	−0.0371	1.5328	1.9204	−0.0328	−0.1021	1.3730	1.3045	0.0065
AR2	−0.0723	1.9782	1.9860	−0.0474	0.0243	−0.1931	−0.2634	0.0029
AR3	−0.0298	1.0366	1.2142	−0.1412	−0.1153	1.9177	1.8285	0.0004
Average slope	−0.0464 m.s.	1.5159*	1.7069*	−0.0738 n.s.	−0.0644 n.s.	1.0325 n.s.	0.9565 n.s.	0.0033 n.s.
	AR-NB, generations 3–40 (of AR)					AR-NB, generations 5–35 (of AR)		
	A1R	F1–7	F8–12	RF	RM	FDT	MDT	VIAB
AR1-NB1	−0.0272	0.7559	0.9647	0.0792	0.0188	−0.0112	0.3297	0.0030
AR2-NB2	−0.0710	1.3255	0.8159	0.1928	0.1616	−2.1711	−1.8052	0.0014
AR3-NB3	−0.0183	0.7631	0.3367	−0.0651	−0.0271	−0.4300	0.0973	−0.0072
Average slope	−0.0388 n.s.	0.9482*	0.7058 m.s.	0.0690 n.s.	0.0511 n.s.	−0.8708 n.s.	−0.4594 n.s.	−0.0009 n.s.

Adult traits: age of first reproduction (A1R); early fecundity (F1–7); peak fecundity (F8–12); female starvation resistance (RF); male starvation resistance (RM). Juvenile traits: female and male development time (FDT and MDT); viability (VIAB).
n.s., $P > 0.1$; m.s., $0.05 < P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

F-test with all replicate data points included; $P < 0.05$, for *t*-test). These two sets of laboratory populations were founded synchronously from different wild source populations, suggesting an effect of their population of origin. Figure 6 shows that AR populations evolved at a slower rate than TW populations, relative to their initial differentiation.

Discussion

Initial adaptation

It is clear from our results that some functional characters evolutionarily respond to domestication in a predictable fashion, improving in an intuitively expected way, particularly in the early generations of the process. Fecundity traits in our laboratory show clear initial improvement during domestication, a pattern that is qualitatively consistent among all domesticated *D. subobscura* populations that we have studied, regardless of time or location of initial sampling (Matos *et al.*, 2000b, 2002 and the present study). During the first 50 generations of domestication in our NW, TW and AR sets of populations we observed 70 %, 79 % and 60 % increases in early fecundity. These results are in general agreement with those reported by Gilligan & Frankham (2003), who also found rapid adaptation to a novel environment over multiple generations in *Drosophila*, as well as the comparative findings of other laboratories, particularly for early fecundity (Sgrò & Partridge, 2000; Hercus & Hoffmann, 1999).

But other characters that might be expected to improve with domestication do not do so. Developmental rate and juvenile viability showed no clear pattern of improvement in the present study. Starvation resistance does not show consistent directional improvement in the present data. In an earlier study, we observed significant early improvement in the NW populations (Matos *et al.*, 2002), but the present results suggest overall decline. In the present study, we found significant improvement over the first 40 generations of domestication in our TW populations, but no significant improvement in our AR populations. This lack of consistency between populations may be a result of genetic trade-offs only becoming observable at a later phase of adaptation (see Service & Rose, 1985; Matos *et al.*, 2000a, 2002; but see Sgrò & Partridge, 2000; Hoffmann *et al.*, 2001 for a different view). However, it is worth pointing out that the

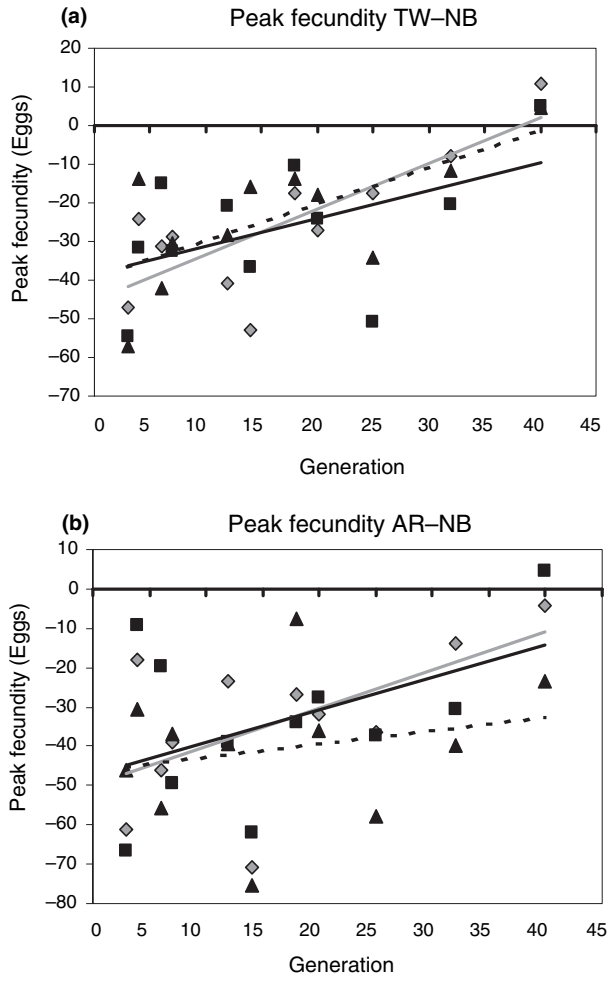

Fig. 5 Peak fecundity evolutionary trajectories for TW minus NB and AR minus NB. (a) Peak fecundity TW-NB, Generations 3–40; (b) Peak fecundity AR minus NB, Generations 3–40. Grey, diamonds, full line – first replicate population (AR or TW); Black, squares, full line – second replicate population (AR or TW); Black, triangles, dashed line – third replicate population (AR or TW).

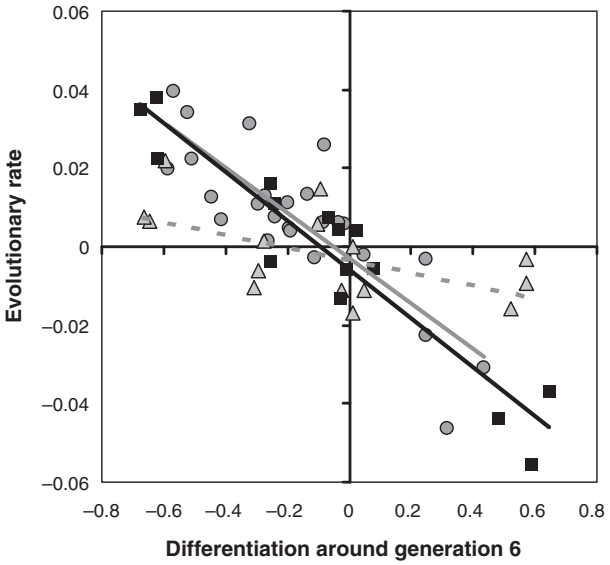

Fig. 6 Linear dependence of evolutionary rate on early differentiation for each set of populations. Each data point of the plot is defined by the early differentiation and the corresponding slope of each evolutionary trajectory (see *Materials and methods*). Grey, circles, full line – NW populations; regression model: $y = -0.0576x - 0.0028$ ($R^2 = 0.6922$, d.f. = 23; $P < 0.00001$). Black, squares, full line – TW populations; regression model: $y = -0.0622x - 0.0058$ ($R^2 = 0.8968$, d.f. = 13; $P < 0.00001$). Grey, triangles, broken line – AR populations; regression model: $y = -0.0164x - 0.0031$ ($R^2 = 0.3414$, d.f. = 13, $P < 0.025$).

response of starvation resistance to domestication also shows disparity of results between laboratories, e.g. decreased starvation resistance in a study by Hoffmann *et al.* (2001), whereas Griffiths *et al.* (2005) observed an increased performance for this trait.

The lack of apparent directional response to selection by some functional characters, but not others, was somewhat unexpected, but it continues a tradition in experimental evolution. It is now clear that adaptation involves an unsynchronized mosaic of evolutionary changes. The source of such differences between characters is not yet known, but it is unlikely to be the absence of genetic variation because almost all characters respond to laboratory selection, when it is applied determinedly (see Prasad & Joshi, 2003; Rose *et al.*, 2004).

Selection response plateaus

In addition to our expectations concerning the characters that should respond under domestication, we also intuitively expected that the response to the selection pressures of domestication would slow progressively. In *Drosophila*, Gilligan & Frankham (2003) found a slowing down of the rate of adaptation to captivity as measured by a competitive index after 87 generations in the lab. Evolutionary plateaus were also observed in the classic Lenski studies of domestication in *E. coli* (e.g. Lenski & Travisano, 1994; Lenski, 2004).

We have evidence for a similar plateau pattern in the fruit flies that we study. For the long-established NB populations, least-squares linear regression of early and peak fecundity over generations 94–176 (see Table 1) shows no significant improvement under continuing domestication.

NW early fecundity presents a progressive drop in the rate of improvement throughout domestication, giving rise to a good fit to a log-linear model, in contrast with previous studies with a smaller number of generations (Matos *et al.*, 2002). For peak fecundity, there is also the suggestion of a slowing down in the evolutionary response of NW populations (see Fig. 3). We interpret this slowing as evidence for the deceleration of functional

improvements after more than 100 generations of domestication, at least for fecundity, if not for all functional characters (see Table 1).

By contrast, Lenski and his colleagues (e.g. Lenski & Travisano, 1994; Cooper & Lenski, 2000) found that *E. coli* took around 5000 generations to reach a plateau. This disparity in the rate of slowing of adaptation between laboratory experiments using *Drosophila* and *E. coli* could be because the *E. coli* experiments relied exclusively on the occurrence of new mutations as each population derived from a single clone (cf. Elena & Lenski, 2003), whereas in outbred populations of *Drosophila* standing genetic variation is almost certain to be the primary factor in the initial response to domestication.

Inbreeding depression and genetic drift

The effect of inbreeding is well-known to be a reduction in the average value of functional characters. In the populations studied here, census population sizes vary from 600 to 1200. Effective population sizes were probably not more than 600, from our unpublished estimates. Over the 176 generations of NB culture, assuming a steady effective population size of only 500, the NB populations can be expected to become about 16 % inbred (see Falconer & Mackay, 1996), assuming no countervailing selection. The starvation resistance and developmental time characters were not apparently selected on very strongly during domestication. It might thus be expected that these traits would show a decline in our longer established NB populations as a result of inbreeding depression. Nevertheless, our data do not show that starvation resistance or developmental rate declined over the last 82 generations of domestication in the NB populations. This suggests either a weak phenotypic effect of inbreeding or countervailing selection against deleterious alleles.

Genetic drift can in principle progressively increase the between-line variance among domesticated populations. But between-line variances either decrease or show little directional change in our results. This again suggests that finite population size effects have been small relative to the statistical power of our experiments.

Strict selective breeding processes and maintenance as small-sized populations have predictably led to inbreeding depression during the domestication of most of our commensals. But inbreeding depression may vary considerably from species to species, population to population, trait to trait (Frankham *et al.*, 2002), and is expected to be more severe under stressful environmental conditions (Hedrick & Kalinowski, 2000). We have used moderately large populations in order to highlight the role of selection in domestication. If we had used effective population sizes an order of magnitude smaller, we would probably have detected the action of genetic drift and inbreeding depression (cf. Montgomery *et al.*, 2000; Woodworth *et al.*, 2002; Rodriguez-Ramilo *et al.*, 2006).

Effects of source wild population

There was a significant disparity between the initial rates of response to domestication as a function of initial differentiation among NW, TW and AR populations for the range of characters we have studied (Fig. 6). It is notable that the NW and TW groups are not significantly different from each other, but both are significantly different from the AR populations. The NW and TW populations were sampled from Sintra, Portugal, though during different years, whereas the AR populations were founded from flies collected at Arrábida, Portugal, on the other side of the Tagus river. These results suggest that there may be significant effects of foundation on the evolutionary response to domestication, such that populations founded with samples from different wild populations respond at a different rate, when the magnitude of initial differentiation is eliminated from the analysis.

We are encouraged that three separate samples from the same wild population of *D. suboscura* over a number of years give remarkably similar results over all. Natural selection in the laboratory can apparently be strong enough to override sampling effects over a short period of time from one wild source population, yet sensitive enough to distinguish differences in domestication among populations founded from different source populations. Nevertheless, this finding of course does not allow ready inferences concerning the adaptive process in the wild populations from which samples are taken (cf. Sgrò & Partridge, 2000 vs. Matos & Avelar, 2001; Matos *et al.*, 2004).

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Chapter 3.

Experimental Domestication

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1. Introduction

Many millennia before we understood the basic laws governing biological evolution, we bred our commensal species to our liking, whether for economic or leisure purposes. A range of species from plants to animals were thereby domesticated. In a sense, the longest-running experimental evolution projects are those of domestication, and they have produced an astonishing variety of animal breeds and plant varieties. Naturally enough, Darwin used pigeon and dog cases of domestication to illustrate the capacity of selection to produce evolutionary change in *The Origin of Species* (1859), and then later expanded greatly on this theme in the volumes that he devoted specifically to domestication (Darwin, 1883).

Domestication does not necessarily imply selection directed toward a single goal. In its broader sense, it means evolutionary change when wild populations are maintained in environments controlled, or at least strongly shaped, by human choices. There are several features of such environments that make the study of domestication interesting from the standpoint of evolutionary biology. Domesticated populations suffer more or less drastic changes in population structure, including size. The environment of domesticated populations is more stable than that of the wild, with a reduction in predation and inter-specific competition. Relaxed selection may arise for a wide range of traits. But for other traits, selection may be greatly heightened, sometimes as a result of human intent, but sometimes not. Changes in age-structure, abiotic factors from temperature to nutrients, available space, and so on may lead to significant changes in the components of fitness in domesticated populations. Domestication may thus be a cause of adaptive changes that are well worth analyzing.

Research in experimental evolution often entails the imposition of new selection regimes. These new selection regimes eventually lead to divergent evolution between the populations subject to them and control populations, the latter often being maintained under the antecedent selection regime imposed on the populations directly ancestral to the populations that are subjected to the new selection regime(s). The basic expectation is that sustained directional changes in the phenotypes of the populations subject to the new form of selection, when measured relative to the control populations, can be explained by the imposition of the new selection regime. Replicate populations can test whether such directional changes could be due to genetic drift, which is not

expected to produce sustained directional changes on average (Rose et al., 1996).

Drosophila is of course one of the commonly used organisms in experimental evolution. Studies of laboratory natural selection in *Drosophila* have characterized the evolution of populations subject to different densities (e.g. Mueller et al., 1993), demographic regimes (e.g. developmental rate, see Chippindale et al., 1997; age at reproduction, see Leroi et al., 1994; Luckinbill et al., 1984; Partridge and Fowler, 1992; Roper et al., 1993; Rose, 1984), several stresses (e.g. starvation resistance, see Chippindale et al., 1996; Harshman et al., 1999; Rose et al., 1992; desiccation resistance, see Folk and Bradley, 2005, Gibbs et al., 1997, Hoffmann and Parsons, 1993; see a brief review in Hoffmann and Harshman, 1999), different temperatures (Kennington et al., 2003; Santos et al., 2004, 2005), and so on (see Prasad and Joshi, 2003; Chippindale, 2006, for reviews).

One of the goals of such studies is to characterize the potential of populations to respond directly to selection. By now, it is apparent that most *Drosophila* characters will respond significantly to direct selection. Of greater interest, therefore, is the pattern of indirect response to selection. Sometimes the aforementioned studies have revealed declines in functional characters that are not the target of selection, suggesting the presence of trade-offs or, less plausibly for outbred populations, genetic correlations due to linkage disequilibrium. But such antagonistic indirect responses to selection are not the only possibility. At the start of adaptation to a novel environment, genotype x environment interactions are expected to entail significant positive genetic covariances among life-history traits (e.g. Chippindale et al., 2004; de Jong, 1993; Matos et al., 2000a; Service and Rose, 1985; Stearns et al., 1991).

Many organisms besides *Drosophila* have been studied with the same basic principles and goals: microorganisms (see Elena and Lenski, 2003 for a review of studies of adaptation in microorganisms), vertebrates in the wild (e.g. Reznick and Ghalambor, 2005), other insects (e.g. *Tribolium*, see Wool, 1987, for an example), *inter alia.* The long-term evolutionary studies of adaptation in *Escherichia coli* by Lenski and his collaborators are particularly noteworthy for the large number of generations that they commonly examine (e.g. Lenski, 2004). Nevertheless, outbred *Drosophila*, like other sexual diploid organisms that have not been inbred, have the advantage of abundant standing genetic variation. This genetic variation is expected to be most abundant in large natural populations or in laboratory populations at the moment of their

foundation from wild samples, since these samples will not have lost genetic variability due to either genetic drift with small population sizes or intense directional selection during initial domestication. This is one reason why studies of the evolutionary domestication of *Drosophila* are of interest, as they allow us to characterize the evolutionary dynamics of local adaptation in populations with considerable genetic variation at the start of selection.

Studies involving convergent evolution in *Drosophila* are much less abundant than studies of divergent evolution. Reverse evolution experiments have been done in lines previously derived from a common ancestor by divergent selection, where the return to the ancestral state is tested when the initial environmental conditions are resumed (e.g. Graves et al., 1992; Passananti et al., 2004; Service et al., 1988; Teotónio and Rose, 2000; Teotónio et al., 2002, 2004). Such studies allow us to address the importance of the evolutionary history of populations as a determinant of their capacity to return to ancestral states. In general, convergent evolution among populations subjected to a common selection regime is the normal intuitive expectation, even though most studies are based on comparative approaches that lack detailed evolutionary trajectories (but see below).

Detailed studies of domestication in *Drosophila* have appeared relatively recently in the scientific literature (e.g. Frankham and Loebel, 1992; Gilligan and Frankham, 2003; Griffiths et al., 2005; Hercus and Hoffmann, 1999a,b; Hoffmann et al., 2001; Krebs et al., 2001; Latter and Mulley, 1995; Matos et al., 2000a, 2002, 2004; Reed et al., 2003; Sgrò and Partridge, 2000; Woodworth et al., 2002). Most of these studies indicate that adaptation occurs during domestication, as revealed by improvement in one or several life history traits measured under the conditions of laboratory culture (Frankham and Loebel, 1992; Gilligan and Frankham, 2003; Hercus and Hoffmann, 1999a, b; Latter and Mulley, 1995; Matos et al., 2000a, 2002, 2004; Sgrò and Partridge, 2000; Woodworth et al., 2002). But there are some disagreements among the authors of such studies (e.g. Frankham and Loebel, 1992 cf. Gilligan and Frankham, 2003; Latter and Mulley, 1995; e.g. Hoffmann et al., 2001 cf. Griffiths et al. 2005, Matos et al., 2000a, 2002, 2004). In particular, studies that employ a comparative approach (e.g. Frankham and Loebel, 1992; Gilligan and Frankham, 2003; Griffiths et al., 2005; Hoffmann et al., 2001; Latter and Mulley, 1995; Woodworth et al., 2002) have often reached different conclusions from studies of evolutionary trajectories

(Krebs et al., 2001; Matos et al., 2000a, 2002, 2004). This will be a major theme of this review. Another controversy concerns the use of long-established laboratory populations to test several evolutionary theories (see Harshman and Hoffmann, 2000; Hoffmann et al., 2001; Linnen et al., 2001; Promislow and Tatar, 1998, Sgrò and Partridge, 2000). Finally, another common disagreement in the literature concerns the genetic mechanisms that cause the decline of some traits during laboratory domestication, specifically mutation accumulation, inbreeding depression, and genetic trade-offs (e.g. Bryant and Reed, 1999; Frankham, 2005; Hoffmann et al., 2001; Latter and Mulley, 1995; Sgrò and Partridge, 2000; Shabalina et al., 1997; Woodworth et al., 2002). We will discuss these issues in light of our own results below.

Real-time studies of evolutionary trajectories during domestication test the assumption of convergence, as well as allowing the experimenter to tackle such important issues such as the repeatability of the evolutionary dynamics of adaptation, the importance of founder effects in the process of laboratory adaptation, the effects of long-term evolution in the laboratory, etc. The study of evolutionary domestication is particularly useful when it uses as starting populations different collections from the wild, samples that are expected to be a highly variable source of founders each time a study is conducted. By following the dynamic changes that occur within domesticating populations it is possible to infer evolutionary rates and define evolutionary patterns directly. Though some short-term real-time studies of evolutionary trajectories have appeared in the *Drosophila* literature (e.g. Hercus and Hoffmann, 1999a; Krebs et al., 2001), to our knowledge ours are the longest-term real-time studies of domestication in a sexual species that have been published to this point (Matos et al., 2000, 2002, 2004; Simões et al., 2007see below).

In this chapter we start by reviewing our own results, extended to a few more generations relative to Simões et al. (2007), and then review other relevant studies, particularly focusing on the points of disagreement between laboratories already alluded to above. We end with suggestions for future studies.

2. Evolutionary Domestication: real-time studies in *Drosophila*

Since 1990 we have studied the evolutionary changes that occur during laboratory adaptation in the model organism *Drosophila subobscura*. However, we will focus here on experiments that were started in 1998, 2001, and 2005, using the population first domesticated in 1990 as a point of reference. In order to illustrate the type of results that we have obtained, we outline our results for just two adult traits, early fecundity and female starvation resistance, though we have studied a number of other functional characters.

2.1. Populations and experimental designs

All our populations were founded from wild samples collected over one to several days using fermented fruit in traps. The first foundation was done in 1990, in an Adraga pinewood in Sintra, Portugal, from which we established our reference laboratory population for our subsequent studies of domestication (the 'NB' populations). Later, in 1998 we collected flies from the same natural location, from which we established a second set of laboratory populations (labelled from here on 'NW'). In 2001, we founded two new sets of populations, one from collections again in Adraga, Sintra (called 'TW') and another from a pinewood in Arrábida, some 50 km from Adraga, on the other side of the Tagus river (called 'AR')- see figure 1. The collections from Arrábida and Sintra were made synchronously, allowing us to follow the evolutionary dynamics of the two sets of populations in parallel simultaneous assays, with the same number of generations in the laboratory, an ideal situation for studying the effects of different wild source populations on the process of domestication.

Each population was split into several replicate populations two generations after the collection of individuals from the wild, with the exception of the long-domesticated population founded in 1990, which was split up into replicates in 1998 at the same time as the then newly-sampled flies were split. We label each replicate by a number. Thus the NW1, NW2, and NW3 are the three populations derived from the "NW foundation" started with wild samples collected in Adraga in 1998.

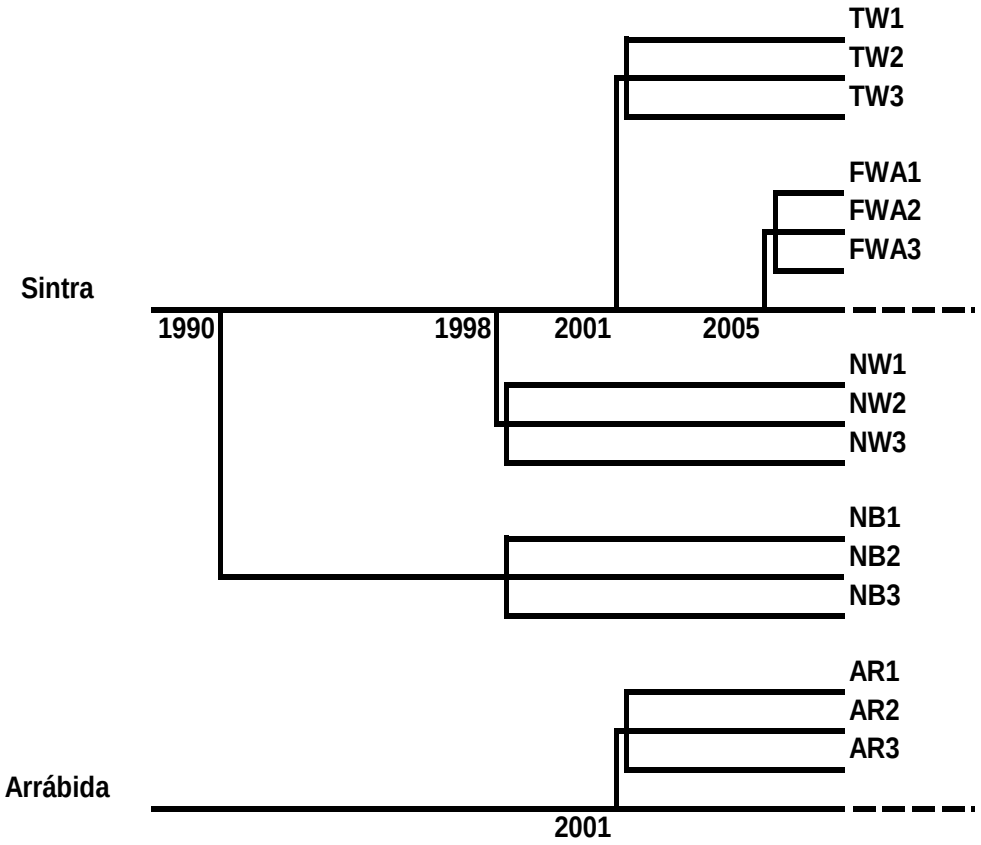

Figure 1. Phylogeny of our 15 laboratory populations, indicating the original wild location. Our reference populations were obtained from the collection of flies of 1990 done in a pinewood of Adraga (Sintra), from which all foundations from Sintra were derived. By the time the last foundation was performed (FWA, 2005) the populations established in 1990, 1998 and 2001 were, respectively, at generations 181, 91 and 45. The foundations of Arrábida and Sintra, in 2001, were carried out synchronously. 3 replicate populations were derived from each of the 5 foundations.

From the moment our laboratory populations were founded, they were maintained in standard conditions, at discrete generations of 28 days, close to the time of peak fecundity in *D. subobscura*, with control of medium, temperature, and population density. Our populations were maintained in numerous vials placed in racks within incubators, with care taken to avoid handling differences among populations during both maintenance and assays. Population sizes were typically about 1200 (see details in Matos et al., 2002, 2004; Simões et al., 2007).

Adult assays were done periodically, both on the more recently introduced

populations and the longer established (NB) populations. We will present here data for mean fecundity during the first week of life and female starvation resistance over generations 4 to 94 of the populations founded in 1998 and the corresponding generations 94 to 184 of the longer established populations, as well as generations 3 to 48 of the populations founded in Sintra and Arrábida in 2001, when the longer established populations were in their generations 139 to 184. Since the unit of evolutionary studies is the population and not the individual, our data analysis focuses on the averages of each replicate population using as source of error the heterogeneity among replicate populations.

2.2. Long-term evolutionary domestication

Early fecundity – There was no significant phenotypic trend among the longer established NB populations between generations 94 and 184, while the NW populations founded in 1998 showed a significant improvement in performance relative to the NB controls between their generations 4 and 94 (Figure 2, average slope=0.411, *t*-test, p=0.02). A log-linear trend is even more significant (p=0.007, data not shown). Altogether these data indicate a clear, though not very quick, process of adaptation in early fecundity.

Female starvation resistance – In figure 3 we present the changes in female starvation resistance shown by the long-established populations between generations 94 and 184. Contrary to the data on fecundity, these populations show a significant decline in female starvation resistance (*t*-test, p=0.03) with an average slope close to -0.05. This corresponds to a decline of around 0.11% per generation, and a decline of about 10% during the entire period**.**

Ehiobu et al. (1989) found that viability in *Drosophila melanogaster* decreased by about 0.96% for every one (1) per cent increase in the inbreeding statistic, F. In our

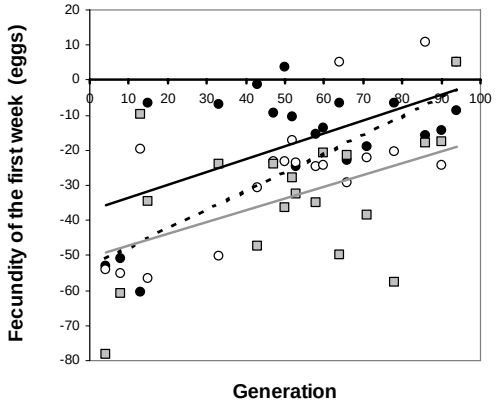

Figure 2. Fecundity during the first week of life in the populations founded in 1998 (NW) relative to the longer-established (NB) populations. Each data point is the difference between the average absolute values of each population and the same numbered longer established population. Replicate population 1: black circles, full black line; replicate population 2: open circles, broken line; replicate population 3: grey circles, grey line. All analyses of linear regressions used the individual slopes as data points in a *t*-test. NW populations show a steady increase in early fecundity throughout laboratory culture, corresponding to a significant pattern of convergence to longer established reference populations.

case, assuming an effective population size around 500 individuals, we expect between generations 94 and 184 an increase in F value of around 8%, which corresponds to a decrease of 1.2% in female starvation resistance for every one per cent increase in the inbreeding statistic F. These values can thus be explained by inbreeding depression alone. There is no significant temporal change of female starvation resistance in the NW populations founded in 1998 relative to the long-established NB populations (average slope=-0.006, n.s.). Nevertheless, the NW populations also show a significant decline in starvation resistance when absolute values are analysed (average slope=-0.05; *t*-test, $p<0.01$, data not shown).

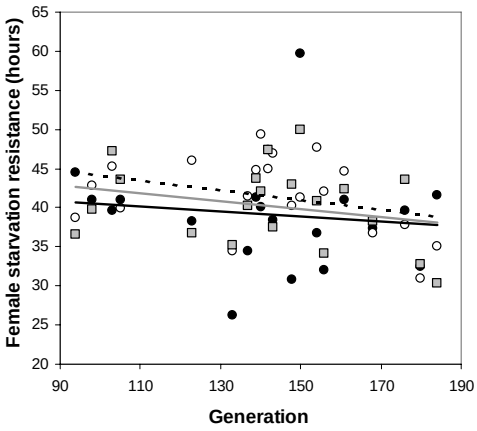

Figure 3. Female starvation resistance in the longer established (NB) populations between generations 94 and 184. Replicate population 1: black circles, full black line; replicate population 2: open circles, broken line; replicate population 3: grey circles, grey line. The analysis used the individual slopes as data points in a *t*-test. NB populations show a significant decline in female starvation resistance with an average slope of –0.05, which corresponds to a decrease of about 0.11% per generation.

2.3. Short-term effects of foundation and repeatability of evolution

Early fecundity – Figure 4 presents the changes in fecundity over the first week of life for the populations founded in Sintra ('TW') and Arrábida ('AR') in 2001, relative to the long-established NB populations. Both regimes show significant linear improvement during the generations under study (average slope=1.27, $p<0.001$ for 'TW'; average slope=1.03, $p=0.024$ for 'AR').

It is interesting to compare the slopes of these 2001 populations with the pattern of adaptation shown by the NW populations founded in 1998, over the equivalent generations 4 and 47. The NW populations had an average slope of 0.76 in that period relative to the reference 'NB' populations, a result not significantly different from that obtained with the populations founded in 2001.

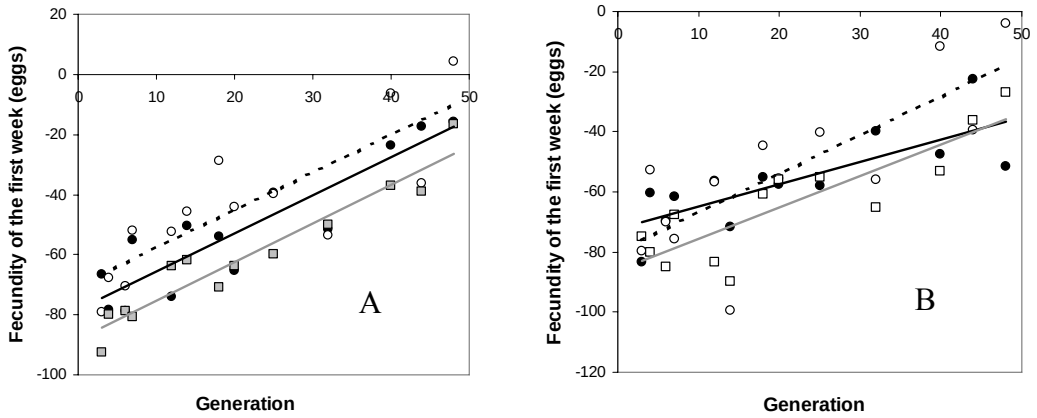

Figure 4. Fecundity of the first week of life in the populations founded in Sintra (A) and Arrábida (B) in 2001, between generations 3 and 48, relative to the longer-established populations. Replicate population 1: black circles, full black line; replicate population 2: open circles, broken line; replicate population 3: grey circles, grey line. The analysis of each set of replicate populations used the individual slopes as data points in a t-test. Early fecundity significantly increases during domestication of both sets of populations. There are no significant differences in evolutionary rate between the two sets of populations.

Female starvation resistance – there is no significant temporal linear increase in starvation resistance among the populations founded in Sintra and Arrábida in 2001, relative to the longer established populations ('TW', average slope=0.114, n.s.; 'AR', average slope=0.124, n.s.; data not shown). Interestingly, the analysis of the temporal changes during the first 40 generations reveals a significant improvement in TW populations, though with a low rate (average slope=0.06, p=0.04; see Simões et al., 2007), which suggests that heterogeneity between replicates with further analysis may have affected the statistical power. As for absolute values, both sets of populations showed a suggestion of a decline during the study, though it was not as clear as we had previously obtained for the NW populations founded in 1998 ('TW', average slope=-0.09, n.s.; 'AR', average slope=-0.08, n.s.). Also, in contrast with the populations founded in 1998, there is no significant improvement of female starvation resistance when considering the first 14 generations, relative to longer established populations. This suggests that any improvement that may occur in the initial period of domestication differs among populations. In view of this result, it is apparent that generalizing from short-term studies of domestication can be misleading. This may

explain some of the disparities in the conclusions of different studies of domestication (see the last section).

2.4. Balance of our studies

In the three studies summarized here there is clear adaptation to the laboratory, in the steady increase in early fecundity. The long-term study of the populations founded in 1998 also suggests that an adaptive plateau is being reached, indicated by a slowing down of the evolutionary rate. Our data indicate that inbreeding depression may play some role in the changes observed under domestication.

One of the odd features of our data is that starvation resistance initially increases and then decreases. This is the expected outcome if the genetic correlation between traits undergoing domestication changes through time, from positive (or less negative) to negative (or more negative) values. The laboratory populations founded from Sintra in 1998 and 2001 show an initial phase with a significant increase in starvation resistance, while more generations show a shift to a negative slope. It is possible that both inbreeding depression and selection act during the evolutionary changes of starvation resistance. The relative importance of these mechanisms of selection and inbreeding may in general change as a function of the initial composition of the population, selective pressures, and how long studies are conducted.

3. Comparative studies of domestication

The comparative method has been increasingly used in experimental evolution. Several studies have used this method to study evolutionary patterns in laboratory adaptation as opposed to the analysis of evolutionary trajectories that we illustrated in the previous section of this chapter.

Here we discuss briefly such comparative studies.

3.1 Static comparisons of long-established *vs.* recently introduced populations

Several studies of laboratory adaptation compare populations that differ in the number of generations in the laboratory with other populations, recently introduced from the wild. These studies do not present data on evolutionary dynamics. In some of these studies the effects of population size, degree of inbreeding, etc. are also analysed.

Hercus and Hoffmann (1999b) conducted a study involving inter-specific hybrids between *Drosophila serrata* and *Drosophila birchii*. This study was short in duration and lacked adequate reference populations, but the results are suggestive. Populations that had been kept in the lab for 17 to 20 generations were compared with populations derived from the same location that had spent just 7 generations in the laboratory. Both fecundity and desiccation resistance were higher in the populations that had been in the lab longer, suggesting that desiccation resistance had increased without a trade-off with fecundity. It is a pity that these authors did not analyse the changes of these traits within each population over multiple generations, as they did for juvenile viability between generations 17 and 30, which showed a temporal increase in performance (Hercus and Hoffmann, 1999a).

Latter and Mulley (1995) conducted a very interesting study in *Drosophila melanogaster* laboratory populations. These authors analyzed the effects of both adaptation and inbreeding on reproductive ability in competitive and non-competitive environments. They compared the performance of populations derived from the same wild source population, but differing in the degree of inbreeding. Comparisons with recently introduced populations were also performed. Long-established populations were superior in competitive ability in the laboratory relative to both recently introduced and inbred populations. Over about 200 generations there was a doubling of competitive fitness even in populations with a population size of 50 during most generations. Comparing differences in performance as a function of the amount of inbreeding, the authors were able to disentangle effects of inbreeding from effects of selection. They concluded that both processes had acted in the inbred populations. Interestingly, fitness differences were minor in a non-competitive environment, obviously indicating the presence of genotype x environment interactions for these characters.

Woodworth et al. (2002) also analyzed the effects of both adaptation and inbreeding during evolutionary domestication. They founded laboratory populations of

Drosophila melanogaster at population sizes ranging from 25 to 500 individuals and compared their performance after 50 generations in the laboratory, both in 'benign' captive conditions and in 'wild' competitive conditions. Several control populations were used in this study, some derived from the same location in later years. In benign conditions, populations of bigger size showed a higher performance while those with the smallest population size performed poorly. In 'wild' conditions, all laboratory populations had a lower performance than the recently derived populations. The authors concluded that both genetic adaptation and inbreeding depression were responsible for the poor performance of laboratory populations in the 'wild' environment.

3.2 Evolutionary dynamics inferred from a comparative approach

In this experimental strategy, populations introduced into the laboratory environment at different times are compared synchronously at different stages of the adaptation process and with this data the evolutionary trajectory of a single population adapting to the laboratory environment is inferred. The assumption is that the evolutionary pattern of the different populations used would be the same if they were compared directly over multiple generations, and so the performance of the most recently founded population will accurately reflect the early stages of adaptation of the previously founded populations. For example, Sgrò and Partridge (2000) compared life-history traits in populations of *Drosophila melanogaster* founded three consecutive times from the same natural location, maintained in either bottles or population cages. The analyses revealed marked changes in some of the traits but few changes in most of them. Differences were found between populations from cages and bottle culture as function of time in the laboratory. Development time increased during laboratory culture, particularly in cages. The authors explained this in terms of increased larval competition in laboratory culture. Early fecundity increased with bottle culture, while late fecundity decreased. However, with cage culture the fecundity patterns were less clear. This was assumed to be due to the truncation of the adult period in bottle culture enhancing the relative focus of natural selection on the early adult period. The authors propose that this led to a decrease in late fecundity by either mutation accumulation or antagonistic pleiotropy.

Using the same three sets of populations and a new one from a recent foundation, Hoffmann et al. (2001) tested the hypothesis that stress resistance is lost during laboratory adaptation. The most-recently founded populations showed higher

starvation and desiccation resistance than the previously founded ones, a result that was interpreted as a marked evolutionary decline in resistance for both stresses during laboratory adaptation. According to the authors, the rapidity of the response ruled out mutation accumulation as a possible explanation for the pattern obtained. They propose that the most likely explanation is that resistance to starvation and desiccation was lost as a correlated response to selection on early fertility, as a result of a negative genetic correlation between stress resistance and fecundity traits.

To investigate the genetic dynamics of adaptation to captivity, Gilligan and Frankham (2003) also used the comparative approach, measuring the fitness of several independently founded populations of *Drosophila melanogaster*, derived from the same natural site in consecutive years, relative to a genetically marked stock. The authors inferred a curvilinear pattern of adaptation, with a 3.33 increase in initial fitness after 87 generations of laboratory adaptation.

Griffiths et al. (2005) studied the effects of laboratory adaptation in *Drosophila birchii* using isofemale lines established from collections made in the same four natural locations over three consecutive years. They concluded that time in laboratory culture influenced evolutionary responses for some traits but not others. For example, there was an increase in starvation resistance and development time in the laboratory lines, while recovering time following a cold shock decreased. On the other hand, heat knockdown resistance and wing size were not affected. The authors argue that collections made in different locations and the use of isofemale lines can overcome the limitations of using a classic comparative approach (e.g. Hoffmann et al., 2001; Sgrò and Partridge, 2000). Nevertheless, the data on development time presented in this study clearly illustrates some of the limitations of this approach, in that the data of one of the sets of lines were quite different from the others. The authors attributed this to changes in the genetic composition of the wild populations.

Although some traits appear to give consistent results across studies (e.g. increased fecundity and development time during laboratory adaptation) others, such as stress resistance, do not. This may not only be due to the different genetic composition of the populations analysed but also to methodological issues (see below).

3.3 Testing comparative methods using trajectory data

We will now test the validity of the comparative approach with our own data, as we now have several sets of populations founded at different times, and know their actual evolutionary trajectories. The question is: can evolutionary dynamics be correctly inferred using comparative data only?

In a recent study, Matos et al. (2004) tested the consistency of results using both the comparative and temporal methods applied to the study of domestication. Although the comparative method proved to be quite accurate for the analysis of robust evolutionary patterns, such as those of fecundity traits, it can lead to problems with less predictable traits. This applies clearly to starvation resistance. Our own studies of real-time evolution suggest that starvation resistance is a trait that has complex evolutionary trajectories during domestication, rendering short-term and comparative studies problematic. It is also a trait that has given disparate results among laboratories in studies that infer evolutionary changes from comparisons among contemporaneous populations. For example, while Hoffmann et al. (2001) found a consistent decline of this trait over generations with laboratory culture, the study by Griffiths et al. (2005) finds an improvement during laboratory adaptation.

We can illustrate this problem using new data that we have collected from a new 2005 foundation from Sintra, the same location where the 'TW', 'NW' and 'NB' populations were derived (see figure 1). At generation 3 after foundation we made our first assay with these more recently founded populations (which we call 'FWA'), as well as 'TW' (at their corresponding 48th generation), 'NW' (in the lab for 94 generations) and 'NB' (the longer established populations, for 184 generations in the laboratory).

The plots for both early fecundity and female starvation resistance are presented in figure 5. In that figure we also plot the data obtained in our previous study of 'TW' populations, when these were in their 4th generation (the earliest assay conducted in that study, involving simultaneous assays of NW and NB populations, by that time in their 50th and 140th generations), using the same methodology.

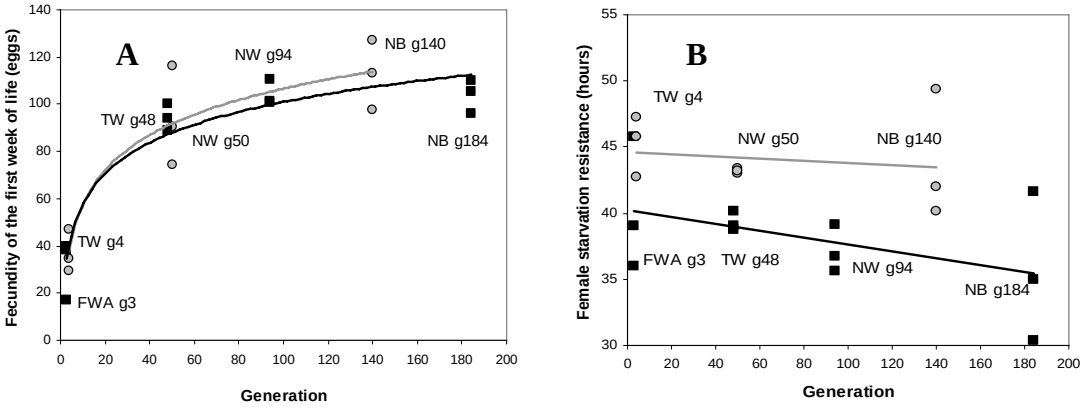

Figure 5. Comparative plots of the values of fecundity of the first week of life (A) and female starvation resistance (B) of independently founded populations as a function of number of generations in the laboratory. Grey circles and grey lines: data from assays done at generation 4 after foundation of TW (NW at generation 50; NB at generation 140); Black squares and black line: data from assays done at generation 3 after foundation of FWA (TW at generation 48; NW at generation 94 and NB at generation 184). Fecundity comparative plots are remarkably similar to those obtained using real-time evolutionary trajectories. These results are also robust among comparative plots using data from different studies. Contrary to this, female starvation resistance shows differences between the two comparative studies: the one using data from TW at generation 4 suggests stability for this trait, while the one for FWA at generation 3 shows evidence for a decline with generations.

Our comparative analysis of fecundity does give similar results to those of our real-time evolution studies, with clear-cut differences between populations as a function of how many generations they have been in the lab, even though they derive from different foundations. There is also robustness of results among the plots using our most recent data and those of the previous study (TW populations at generation 4). But contrary to these fecundity results, starvation resistance shows differences between the two studies: the assay at generation 4 of the TW populations suggests stability of this trait, while the most recent data present evidence for a decline with generations.

These data illustrate one of our points about the limitations of a comparative approach: if the values of the TW populations in their generation 4 were close to the ones presented by our most recent populations (assuming the differences to be purely genetic, which is obviously simplifying) the inferred trend might even be positive. In

fact, the data of an assay done at generation 6 of the TW populations presents such a shift relative to NB values, with TW populations having lower values than these populations, though bigger than 'NW' (see Matos et al., 2004). This does not correspond to any trend in the actual evolutionary trajectories. The problems of a comparative approach are thus clearly revealed by our data.

The differences among comparative studies in the evolution of starvation resistance contrast with the more repeatable patterns obtained with evolutionary trajectories. This suggests that the comparative approach to experimental evolution can yield misleading results. Indeed, the use of contemporaneous populations as 'surrogates' for the evaluation of the phenotypic state of a given population through time (e.g. Frankham and Loebel, 1992; Gilligan and Frankham, 2003; Griffiths et al., 2005; Hoffmann et al., 2001) rests on several untested *a priori* assumptions that may not always apply. For example, it is often assumed that founder effects and random genetic drift during adaptation are negligible (as proposed by Sgrò and Partridge, 2002; but see Matos and Avelar, 2001; Woodworth et al., 2002). Furthermore, comparative studies often lack appropriate reference populations, and this prevents the disentangling of the evolutionary mechanisms involved, particularly in traits exhibiting complex evolutionary trajectories, as our studies of starvation resistance illustrate. We conclude that the comparative approach is not the appropriate tool with which to study the detailed dynamics of evolution.

4. General Issues

4.1 Are lab flies degenerate?

Some have argued that laboratory populations that have been established for many generations are of little use for evolutionary studies (Harshman and Hoffmann, 2000; Linnen et al., 2001; Promislow and Tatar, 1998). Such a view is based, at least in part, on the idea that experimental evolution studies try to extrapolate results from laboratory populations to evolution in the wild. This is not correct. Experimental evolution is about potential genetic changes in response to defined selection regimes. In particular, some have argued that the ability to select for delay of senescence suggests that alleles with different effects at late ages have accumulated in laboratory populations maintained using short generation times, to a much higher extent than would occur in overlapping

generations (Linnen et al., 2001; Promislow and Tatar, 1998). While this is indeed expected, we find this criticism ironic in that, to our view, this is one more reason why populations maintained with discrete generations may be the best material to test for the mechanism of accumulation of mutations (Rose and Matos, 2004). After all, this is one of the important tools of experimental evolution, allowing selection to generate differences between the average phenotypes of populations that permit us to infer underlying evolutionary mechanisms.

More generally, there is no reason to assume that the laboratory environment is not a particular kind of environment, or that laboratory populations are not simply natural populations evolving in that environment (Matos et al., 2000b).

4.2 The problem of complex evolutionary trajectories

The evolutionary trajectories that we have adduced above indicate that starvation resistance is evolving through both selection and drift mechanisms during the domestication of *D. subobscura*. It seems likely to us that these mechanisms might generate non-linear evolutionary trajectories for any particular functional character during longer-term laboratory evolution. How much each of these mechanisms affects the trajectory of a particular character may be rather unpredictable. Novel environments pose difficult evolutionary challenges for both organism and experimenter, challenges that may give rise to genotype x environment interactions that in turn generate novel additive genetic covariances among traits.

How repeatable is evolution? Our data across three different studies of detailed characterization of adaptation to the laboratory suggests general repeatability of evolutionary processes and patterns, though also disparity of results for particular traits. This contingency is apparently related with the relevance of these traits with fitness: early fecundity is clearly a very important fitness component, while this is not expected to occur so much for starvation resistance. Also, short and long term studies can give different results. Our conclusions add to a body of data indicating that though evolution is a global process, its specific outcomes often cannot be generalized (see Rose et al., 2005).

4.3 Application to conservation

Recent interest in characterizing the evolutionary changes of populations from the moment they are brought to the laboratory arises from both their general significance for the study of biological evolution and the need to characterize the specific effects of captivity for the purpose of conservation (Gilligan and Frankham, 2003). Not all agree as to what studies of adaptation during captivity may tell us about the impact of such evolution for conservation purposes. Genotype x environment interactions will limit considerably extrapolations from the laboratory even to zoo and enclosure environments.

We thus certainly cannot extrapolate the findings of evolutionary change in the laboratory to what will occur when populations are re-introduced in the wild (see Shabalina et al., 1997). The evolutionary genetic complexity of functional traits does not allow reliable inference (cf. Reed et al., 2003; Woodworth et al., 2002). As a safe guard, the best strategy may be to avoid prolonged captivity, minimizing concomitant evolutionary changes (Frankham, 1995; Gilligan and Frankham, 2003; Rodriguez-Ramilo et al., 2006; Woodworth et al., 2002 cf. with Shabalina et al., 1997).

4.4 What have we learned about domestication from experimental evolution?

Most studies of evolutionary domestication indicate that adaptation occurs during domestication, as can be inferred from improvement in such traits as juvenile viability (Hercus and Hoffmann, 1999a), early fecundity (e.g. Hercus and Hoffmann, 1999b; Matos et al., 2000a, 2002, 2004; Sgrò and Partridge, 2000 etc), competitive ability (Frankham and Loebel, 1992; Latter and Mulley, 1995) and non-competitive fitness (Woodworth et al., 2002). Some studies differ over the rate of adaptation during captivity (e.g. Frankham and Loebel, 1992 cf. Latter and Mulley, 1995), and short-term studies may be misleading, as we have shown here. Our studies suggest that domestication can involve complex evolutionary trajectories. We have shown that disparate results among studies of domestication may be due to different methodologies, specifically the limitations of a comparative approach (e.g. Gilligan and Frankham, 2003; Griffiths et al., 2005; Hoffmann et al., 2001; Latter and Mulley, 1995) vs. studies of evolutionary trajectories (Krebs et al., 2001; Matos et al., 2000a, 2002, 2004; Simões

et al., 2007). In our view, multiple evolutionary mechanisms can be involved in domestication and their specific relevance will probably vary from case to case.

From an applied standpoint, the study of adaptation to captivity has received progressively more attention in the conservation literature. There is still a substantial need for basic research on the evolutionary and genetic mechanisms relevant to conservation programs, where these mechanisms range from direct and correlated adaptive responses to inbreeding and drift. The experimental study of domestication is a particularly useful vein for such basic research.

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Chapter 4.

How repeatable is adaptive evolution?

The role of geographical origin and founder effects in laboratory adaptation

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Abstract

The importance of contingency versus predictability in evolution has been a longstanding issue, particularly the interaction between genetic background, founder effects, and selection. Here we address experimentally the effects of genetic background and founder events on the repeatability of laboratory adaptation in *Drosophila subobscura* populations for several functional traits. We found disparate patterns of adaptation among laboratory populations derived from independent foundations - either temporally or geographically separated - of wild populations. Furthermore, our results suggest that distinct functional traits can be affected by spatial and temporal factors to different degrees. On balance, although the role of natural selection is evident in demonstrably consistent adaptive responses, our data also support the importance of contingent evolution.

Keywords: Adaptation; Repeatability; Evolutionary Contingency; Founder Effects; Genetic Background; Life History Traits; *Drosophila subobscura*.

Introduction

Evolutionary contingencies can be a source of differentiation among populations (Travisano et al. 1995; Joshi et al. 2003). In particular, differences in adaptive dynamics have been shown between populations with different ancestors that share a common environment in which they undergo subsequent adaptation (Cohan 1984a; Cohan and Hoffman 1986, 1989). Several factors may be involved in such contingent differentiation. Small differences in the course of selection might result in substantially different evolutionary outcomes, particularly in populations studied without good environmental controls. Different genetic backgrounds may explain disparate adaptive responses among populations exposed to the same selective pressures (Cohan and Hoffmann 1989; de Brito et al. 2005), particularly in carefully controlled laboratory studies.

Such differences among genetic backgrounds may specifically involve differences in additive genetic components of variance and covariance, non-additive interactions among loci, or linkage disequilibrium (Lande 1980; Cheverud and Routman 1995; Falconer and Mackay 1996; Roff 2000; Wade et al. 2001; Steppan et al. 2002). These genetic differences may be due both to the past selective history and to random allele frequency changes associated with sampling processes, particularly with bottlenecked population sizes (Bryant et al. 1986; Cheverud et al. 1999; Naciri-Graven and Goudet 2003; Zhang et al. 2004). Furthermore, interactions between directional selection and genetic drift during the evolutionary process might increase the impact of different genetic backgrounds (Cohan 1984b; Cohan and Hoffmann 1989; de Brito et al. 2005).

Experimental evolution studies are particularly well suited to test the relevance of genetic background effects in adaptive evolution, since they allow us to reproducibly measure the adaptive response of replicated populations to defined environmental conditions (Rose et al. 1996; Lenski 2004; Chippindale 2006). The evolutionary responses can be measured relative to control populations, already adapted to the laboratory. Several laboratory experiments have addressed the importance of genetic background effects in *Drosophila* laboratory evolution (Cohan and Hoffmann 1986; Teotónio and Rose 2000; Teotónio et al. 2002; Joshi et al. 2003; see review in Prasad and Joshi 2003; Rose et al. 2004). Overall, these studies found some cases in which

different genetic backgrounds clearly lead to different evolutionary patterns during adaptation to a common environment (e.g. Cohan and Hoffmann 1986; Teotónio and Rose 2000).

Our team has focused on the study of adaptation to the laboratory in *Drosophila subobscura*, studying the evolutionary trajectories of several life history traits in populations collected from different natural locations (see Matos et al. 2000a; 2002). We have already found evidence of variation in adaptive response during the first generations of laboratory adaptation between two sets of populations founded from the same natural location six years apart (Matos et al. 2002). In a subsequent study, we analyzed the evolutionary dynamics of two different sets of laboratory populations derived from synchronous foundations obtained in 2001 from two different natural sites: "AR" populations derived from a wild-caught sample from Arrábida, Portugal, and "TW" populations from Sintra, Portugal (see Simões et al. 2007). This study revealed a clear adaptive response in fecundity-related traits as well as significant differences in the evolutionary dynamics of these two sets of populations over their first 14 generations of laboratory culture, most likely as a result of different initial genetic composition. Our past results thus suggested that the initial phase of life history evolution in a new environment can be quite sensitive to the genetic composition of the wild source populations.

In spite of the clear results observed in that study, we could not specifically determine whether sampling genetic effects due to initial population-foundation itself could account, at least in part, for the different evolutionary dynamics observed between populations from different locations. The random loss of alleles due to a limited number of field-collected individuals is expected to affect the subsequent evolutionary response of populations (James 1971; Powell and Richmond 1974; Reznick and Ghalambor 2005). The magnitude of these effects will depend on the standing genetic variation for fitness-related characters present in the wild population from which samples are taken. Studies of wild populations suggest that there is considerable genetic variation for life history traits (Mousseau and Roff 1987; Merilä and Sheldon 2000). Nevertheless, the importance of possible sampling effects for variation in the laboratory adaptation process is not easily predictable.

In this study we present a broader analysis of the initial stages of laboratory evolution combining data from different sets of populations obtained from collections

across different years (1998, 2001, and 2005) and different geographical locations (Arrábida and Sintra). This analysis aims to test the repeatability of an adaptive process and the impact of contingent factors, such as chance events of prior genetic background – due to either geographical or temporal shifts in the genetic composition of populations.

We also compare the sensitivity of adaptive processes to differences arising from the geographical location of wild source populations (Arrábida *vs.* Sintra) with the impact of sampling effects among populations derived from the same wild source. To tackle these issues we analyze the evolutionary trajectories of several adult life history traits during the first 20 generations of laboratory adaptation in four additional sets of replicated populations collected in 2005, with two independent foundations from each natural location previously sampled, together with data previously collected.

Materials and methods

Foundation and maintenance of the laboratory populations

This study includes data from seven different sets of wild-caught samples of *Drosophila subobscura*. These different sets of populations were founded in the calendar years 1998 (NW populations; see Matos et al. 2002), 2001 (AR and TW populations; see Simões et al. 2007) and 2005 (FWA, FWB, NARA and NARB populations, the new data presented here). Both NW and TW populations were collected from a pinewood near Sintra, Portugal while AR populations were collected from Arrábida, Portugal (see Simões et al. 2007).

The additional foundations reported here were performed in April 2005 and consisted of two independent collections from each of the two previously sampled natural sites: Sintra, Portugal - "FWA" and "FWB" populations; Arrábida, Portugal - "NARA" and NARB". The number of founder females was as follows: FWA – 60; FWB – 75; NARA – 55 and NARB – 68.

All populations were maintained under the same conditions: discrete generations of 28 days, reproduction close to peak fecundity, controlled temperature of 18ºC, with a 12-h L : 12-h D photoperiod. Flies were kept in vials, with controlled adult densities of around 50 individuals per vial and larval densities of around 80 per vial. At each generation, emergences from the several vials within each replicate population

were randomised using CO_2 anaesthesia. Total population sizes were usually between 600 and 1200 individuals (Matos et al. 2000a, 2002; Simões et al. 2007).

Two generations after foundation, each population was split into three replicate populations (e.g. FWA_{1-3} designating the three populations of the regime "FWA"), except the NW foundation, split into five replicates. A set of longer established laboratory populations "NB" was used as a control for all the experimental populations referred above. NB populations were at their 90^{th} laboratory generation when NW populations were founded (Matos et al. 2002). At the time the 2001 AR and TW populations were introduced into the laboratory, the NB populations were at their 136^{th} generation. As for the 2005 collections, the NB populations were at their 181^{st} generation at the time of their initial foundation.

The early adaptation data analyzed in this study corresponds to the first 15 generations of NW culture and the first 20 generations of AR and TW laboratory populations. All new populations cultured from the 2005 foundations were cultured for 21 generations, during which their adaptation to laboratory conditions was also studied by means of phenotypic assays.

Life-History trait assays

In each generation assayed an additional egg collection was made using the same basic maintenance procedure, as described above.

Mated pairs of flies used individuals emerging in the same day, and were formed less than 6 hours after eclosion started (stimulated by the light phase). These pairs were transferred daily to vials containing fresh medium, and the total number of eggs laid per female was counted daily for the first 12 days. After the fecundity assay was performed, each pair of flies was transferred to a vial containing plain agar medium where the number of hours of starvation resistance was measured. Five characters were analyzed: age of first reproduction (number of days between emergence and the day of first egg laying – "A1R"), early fecundity (total number of eggs laid during the first week – "F1-7"), peak fecundity (total number of eggs laid between days 8 and 12 – "F8-12"), female and male starvation resistance (number of hours until death, registered every 6 hours – "RF" and "RM", respectively).

Assays were performed at generations 4, 8, 13 and 15 of NW laboratory culture and at generations 3, 4, 6, 7, 12, 14, 18 and 20 of AR and TW populations. Phenotypic

assays on the 2005 populations were carried out during generations 3, 6, 10, 12, 15 and 21. Sample sizes ranged between 14 and 21 pairs per replicate population.

All assays involved synchronous analyses with NB populations.

Statistical Methods

Analysis of new data

Evolutionary trajectories are presented for the 2005 data. These trajectories were calculated for each life history trait using Type I least-squares linear regressions (Sokal and Rohlf 1995). The regression analysis was carried out using the mean values of traits for each replicate population as the dependent variable and generation number as the independent variable. The evolutionary trajectories of FWA, FWB, NARA and NARB populations were compared using both the actual values for each population as data points and as differences relative to the longer established NB populations (for the same arbitrarily-numbered replicates, e.g. using the difference between the averages of FWA_1 and NB_1 replicates). The latter analysis was used to minimize environmental noise that might obscure the actual evolutionary trends (vid. Matos et al. 2002; Simões et al. 2007). The significance of the linear regression obtained from the trajectory of the data from each set of populations was determined by a *t*-test using the average slope of the evolutionary trajectories of the replicate populations, with the variation of these slopes among replicate populations serving as the sample variation for the purpose of the hypothesis test.

Differences due to natural location and/or sampling effects at each generation assayed were tested by three-level nested ANOVAs for each particular trait using the 2005 data. These analyses included location (fixed), sampling, and replicate population (random). The hierarchical design included the factor sampling nested in location and replicate population nested in both location and sampling. Differences due to natural location and/or sampling were also tested by performing two level nested ANOVAs on the overall evolutionary rates for each trait and population i.e. the slopes of the respective evolutionary trajectories.

These analyses were performed using STATISTICA and EXCEL.

Meta-analysis of all data

The meta-analyses were performed on the overall data from phenotypic assays (using information at the individual level) concerning the initial adaptation process of our laboratory foundations: NW (generations 4-15); AR and TW (generations 3-20); FWA, FWB, NARA and NARB (generations 3-21). A simple linear regression was performed on the data including all foundations, using each life history trait as a dependent variable and generation as the independent variable.

Linear mixed-effects models (Pinheiro and Bates 2000) were subsequently applied. These analyses were performed using the nlme library included in R software version 2.4.1 (http://www.R-project.org). The linear models were fit by Restricted maximum likelihood (REML). Several linear models were applied to the data according to the specific factors being tested. Differences relative to the average of the same-numbered NB replicate population were used as input data for these analyses.

As an example of the formal models used here we consider the case of assessing the significance of different foundations on the response to selection. Let y_{ijk} be the measured response in foundation i (i=1..7), in generation t_j (j=1..m, taking the value of the generation of each assay), in replicate k (k=1..3i). Then the linear model used to model phenotypic values is,

$$y_{ijk} = \mu + \alpha_i + \beta t_j + \gamma_i t_j + b_k + \varepsilon_{ijk},$$

where μ and β measure the average response to selection, α and γ measure the effects of foundation on the intercept and slope of the evolutionary response respectively, b_k is the random effect of replicate population with $b_k \sim N(0,\sigma_1^2)$ and ε_{ijk} is the residual error that is assumed independent of b_k and $\sim N(0,\sigma^2)$. We abbreviate this notation with the following R model statement: trait ~ generation + foundation + generation*foundation. In this analysis, the effect of each foundation was tested relative to NW data, our first foundation done in 1998. Overall, these analyses involved 2359-2454 degrees of freedom for the error source of variation of the generation and generation*foundation terms for all traits analysed; the foundation term involved 16 degrees of freedom for the error.

To test for the effect of either the temporal or the spatial component in the adaptive response of our laboratory populations, more specific linear models were

performed. These included generation, year of sampling (1998, 2001 and 2005, with a total of 19 d.f.), location (Sintra and Arrábida, with a total of 19 d.f.) and their interaction terms as factors. In these linear models, the location factor was treated as both a random and a fixed effect. The two fixed-effects part of the models were: trait ~ generation + year + generation*year, with replicate population nested within location (for location treated as random factor); and trait ~ generation + location + year + generation*year + generation*location, with replicate population as random factor (for location treated as fixed factor). The generation*location and generation*year interaction terms (both involving 2364-2457 d.f. for all traits analysed) are particularly relevant, since they allow us to access the significance of the differences in adaptive response between populations derived from the two different locations or in different years.

The generation*location*year interaction term was also tested using the 2001 and 2005 data to study the temporal variation (across years) of the differences in adaptive response between the two locations sampled (Sintra and Arrábida). The following model was applied: trait ~ generation + location + year + generation*year + generation*location + location*year + generation*location*year - as the fixed part, with replicate population as random. The test for generation*location*year effects involved 2033-2128 degrees of freedom for all traits analyzed.

Results

Laboratory Adaptation

Age of first reproduction

Our results for fecundity traits were analyzed using a one-tailed *t*-test, since we had a clear *a priori* expectation of improvement for these traits during laboratory adaptation. Age of first reproduction showed a significant improvement (indicated by a significant negative slope) for FWB and NARA populations, whether our analysis used actual values or data standardized relative to the long-established NB laboratory populations. FWA populations only showed a significant improvement when analysing actual values, while NARB populations did not show significant trends in either analysis (see Table 1).

Early fecundity (days 1 to 7)

Early fecundity showed significant improvement in all populations using both actual values and after standardization with NB data, with the sole exception of the NARB populations, which did not show a significant linear trend in data standardized using the NB populations (see Table 1 and Fig. 1). However, these NARB populations did show a significant linear trend using their data without standardization.

Peak fecundity (days 8 to 12)

Both FWA and FWB populations showed improvement in peak fecundity using the raw data for their evolutionary trajectories. After standardization with respect to NB data collected in parallel, no significant linear trends were obtained for this trait (see Table 1). This result is due to a significant linear trend of the NB populations during this period, probably due to environmental effects. Given our careful experimental design these environmental effects are expected to have affected similarly all populations analyzed. From this we conclude that no net response occurred for this trait in either FWA or FWB populations.

Starvation resistance

Our results for starvation resistance were analyzed using a two-tailed *t*-test, since in this case we had no *a priori* expectation regarding the direction of evolutionary trajectories for this trait. Both female and male starvation resistance showed a general lack of significant directional change among the evolving populations. The only exception was a significant positive linear trend for male starvation resistance with respect to actual values in FWB populations (see Table 1).

Geographical Location *vs.* Sampling Effects in Adaptive dynamics

Evolutionary trajectories

We directly tested for the importance of geographical location (Sintra *vs.* Arrábida) and sampling genetic effects (two foundations, derived from independent collections, per location) in the evolutionary trajectories of laboratory populations founded in 2005. We first applied a three level nested ANOVA with location, sampling and replicate populations as hierarchical levels to the data of the assays performed during the first 21 generations. A lack of significant differences due to either geographical or sampling

genetic effects was observed across all generations (data not shown). A two level nested ANOVA was then performed for each life-history trait studied using as data the evolutionary rates of each replicate population. These analyses revealed that the evolutionary rates were not significantly different, either between locations or between foundations within locations (i.e. due to sampling effects), for any of the life history traits (data not shown).

Pooled analyses of evolutionary trajectories

Given that the analyses just described did not show any significant differences between collections from the same natural site, the evolutionary trajectories were reanalyzed using the pooling of data from the two collections of the same geographical location (i.e. grouping FWA with FWB and NARA with NARB, and applying t-tests with 5 degrees of freedom in each case). For age of first reproduction and early fecundity a significant improvement was obtained for the pooled data both from Sintra (FW populations) and Arrábida (NAR populations), either with actual values or standardizing with NB data. As for peak fecundity, a significant linear trend was obtained for both FW and NAR populations using actual values, contrasting with the previous analysis that showed non-significant patterns for the two Arrábida foundations when analyzed separately. The pooled analysis also revealed a significant linear increase in male starvation resistance for FW populations using both actual values and data standardized relative to NB (data not shown). Female starvation resistance did not show any significant trend, in agreement with the analysis of separate foundations.

Paired *t*-tests performed on the slopes of the evolutionary trajectories of the 6 FW and NAR populations did not show significant differences between these two sets of populations for any life history trait, except for a significantly higher slope for FW male starvation resistance.

Table 1. Slopes of least squares linear regressions of the indicated traits for each FWA, FWB, NARA and NARB replicate population. The analysis of each set of populations used the individual slopes as data points in a *t*-test; at the bottom line for each set of populations the average slope of the linear model and the significance level of the *t*-test are presented.

	FWA populations, generations 3-21					**FWB populations, generations 3-21**				
	A1R	F1-7	F8-12	RF	RM	A1R	F1-7	F8-12	RF	RM
Replicate Population 1	-0.0869	3.4837	4.2384	0.3444	0.4950	-0.0987	3.6670	3.7569	0.4186	0.5091
Replicate Population 2	-0.0352	1.9489	2.2875	0.4474	0.4264	-0.1348	3.4570	5.0949	0.0731	0.2744
Replicate Population 3	-0.0897	3.1183	2.4313	-0.5038	-0.0471	-0.0461	3.3140	3.5824	-0.1705	0.3147
Average slope	-0.0706*	2.8503*	2.9857*	0.0960n.s.	0.2914n.s.	-0.0932*	3.4793***	4.1447**	0.1071n.s.	0.3661*
	NARA populations, generations 3-21					**NARB populations, generations 3-21**				
	A1R	F1-7	F8-12	RF	RM	A1R	F1-7	F8-12	RF	RM
Replicate Population 1	-0.0431	2.3663	1.4058	-0.0825	0.3048	-0.1142	4.2836	5.2749	0.3993	0.3067
Replicate Population 2	-0.0547	2.5066	0.6051	0.1644	0.2751	0.0349	1.8365	1.5240	0.4320	0.0888
Replicate Population 3	-0.0872	3.7065	4.1144	-0.2736	-0.0180	-0.0351	3.0009	2.0309	-0.1366	-0.1070
Average slope	-0.0617*	2.8598*	2.0418m.s.	-0.0639n.s.	0.1873n.s.	-0.0381n.s.	3.0403*	2.9433m.s.	0.2316n.s.	0.0962n.s.
	FWA-NB, generations 3-21					**FWB-NB, generations 3-21**				
	A1R	F1-7	F8-12	RF	RM	A1R	F1-7	F8-12	RF	RM
Replicate Population 1-NB1	-0.1309	2.5148	2.7081	-0.0245	0.2636	-0.1426	2.6981	2.2265	0.0497	0.2776
Replicate Population 2-NB2	-0.0247	0.6174	-0.4843	0.0731	0.2328	-0.1244	2.1255	2.3230	-0.3012	0.0809
Replicate Population 3-NB3	-0.0993	1.7740	-0.9952	-0.8098	-0.0648	-0.0556	1.9697	0.1558	-0.4765	0.2969
Average slope	-0.0850m.s.	1.6354*	0.4095n.s.	-0.2537n.s.	0.1439n.s.	-0.1075*	2.2644**	1.5684m.s.	-0.2427n.s.	0.2185m.s.
	NARA-NB, generations 3-21					**NARB-NB, generations 3-21**				
	A1R	F1-7	F8-12	RF	RM	A1R	F1-7	F8-12	RF	RM
Replicate Population 1-NB1	-0.0870	1.3974	-0.1245	-0.4514	0.0734	-0.1581	3.3147	3.7445	0.0304	0.0753
Replicate Population 2-NB2	-0.0443	1.1751	-2.1668	-0.2099	0.0815	0.0454	0.5051	-1.2479	0.0577	-0.1048
Replicate Population 3-NB3	-0.0967	2.3622	0.6878	-0.5796	-0.0358	-0.0447	1.6566	-1.3956	-0.4426	-0.1248
Average slope	-0.0760*	1.6449*	-0.5345n.s.	-0.4136m.s.	0.0397n.s.	-0.0525n.s.	1.8255m.s.	0.3670n.s.	-0.1182n.s.	-0.0514n.s.

Life-History traits: Age of first reproduction (A1R); Early fecundity (F1-7); Peak fecundity (F8-12); Female starvation resistance (RF); Male starvation resistance (RM). Levels of significance: n.s., $P>0.1$; m.s., $0.05<P<0.1$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$;

Fecundity Traits: one tailed *t*-tests; Starvation Resistance: two tailed *t*-tests.

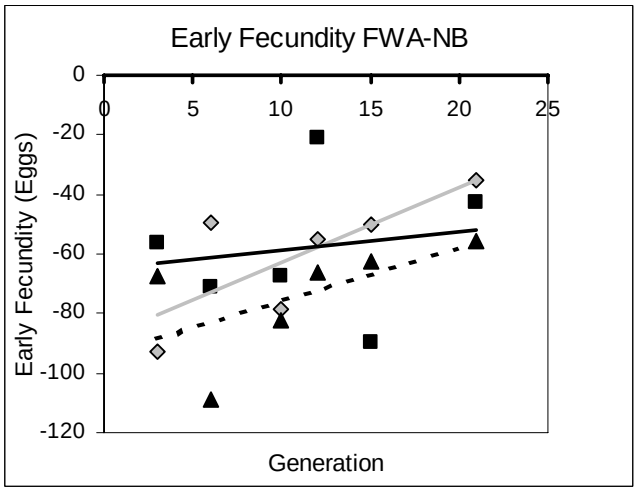

Figure 1A

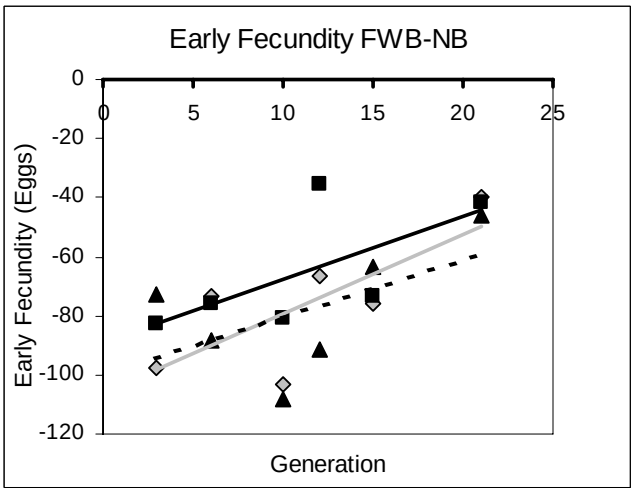

Figure 1B

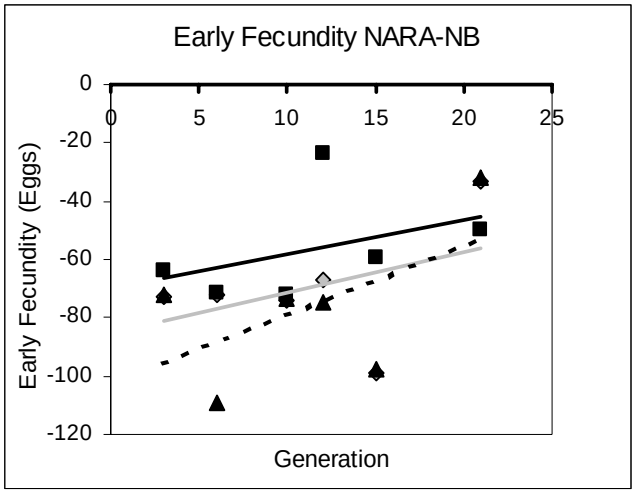

Figure 1C

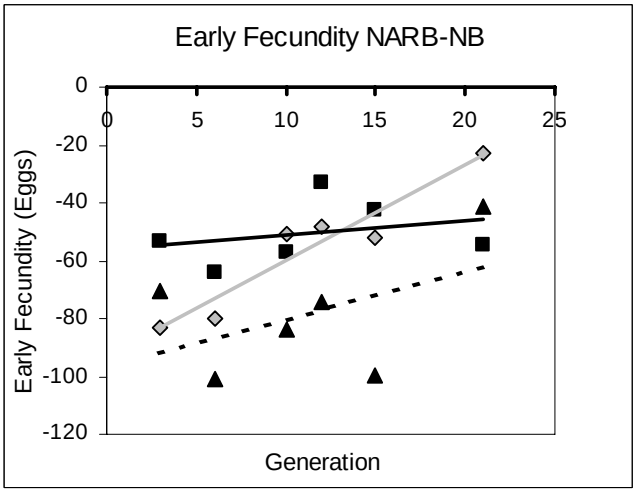

Figure 1D

Figure 1. Early fecundity evolutionary trajectories for the four 2005 foundations between generations 3 and 21. (A) FWA-NB (Sintra); (B) FWB-NB (Sintra); (C) NARA-NB (Arrábida); (D) NARB-NB (Arrábida). Grey, Diamonds, full line – first replicate population; Black, Squares, full line – second replicate population; Black, Triangles, dashed line – third replicate population.

Meta-analysis of All Data

Age of first reproduction

A simple linear regression including data from all foundations showed a significant negative slope indicating an evolutionary response in this particular trait, corresponding to a faster maturation with generations. An initial linear mixed effects analysis was applied to test for differences that can potentially arise from different independent foundations (see Fig. 2a). When testing the effect of each foundation relative to NW data, only the comparison with the TW foundation revealed a significant interaction generation*foundation. Thus, age of first reproduction evolutionary response differed significantly between these two foundations.

To further investigate the effects (temporal and/or spatial) associated with the variation in evolutionary response other linear models were performed. These included generation, year of sampling, location – considered as fixed and random - and their interaction terms (see Materials and Methods). The age of first reproduction values averaged over all assayed generations were significantly different across locations and years of sampling (significant year and location terms). The interaction term generation*location was also significant, indicating that the response to laboratory selection was different among locations for this character (see Fig. 2a). When treating location as a random effect, significant results were also obtained for the year factor. This indicates that the age of first reproduction values averaged over all assayed generations differed significantly among years of sampling.

Early fecundity (days 1 to 7)

A simple linear regression involving all foundations indicated a clear improvement with time (significant generation effect). This response differed between NW and other foundations as revealed by significant generation*foundation interactions (see also Fig. 2b).

Significant generation*year interaction factors were obtained for both models performed for this trait (using location as either a fixed or a random effect). This indicates that the evolutionary response varied predominantly between different years of sampling for this trait.

A significant year (2005) factor was significant indicating differences in average early fecundity values between the 1998 and 2005 populations.

Peak fecundity (days 8 to 12)

This trait presented a clear evolutionary response considering all foundations. A significant generation*foundation interaction was obtained when comparing NW (Sintra) foundation relative to the AR, NARA and NARB (Arrábida) foundations. This suggests that the differences in the evolutionary responses observed across foundations are likely to be associated with geographical (i.e. location) effect (see also Fig. 2c).

Indeed, analysis of peak fecundity showed varying evolutionary response between locations for this particular trait, as indicated by a significant generation*location interaction term, defining location as a fixed effect in the linear model.

Assuming location as a random effect in the linear model, the term year (2001) was significant, indicating differences in the peak fecundity average values (across all generations assayed) between the NW populations (1998) and AR and TW populations (2001). The generation*year (2005) interaction term was also significant, suggesting differences between the evolutionary response of the 1998 populations (NW) relative to the 2005 foundations (FWA, FWB, NARA and NARB).

Female starvation resistance

A simple linear regression on the female starvation resistance values of all foundations showed a general decline during laboratory culture (significant generation effect; negative slope). This trait revealed significant generation*foundation interactions, indicating clear differences in the evolutionary dynamics between NW and the other foundations (see Fig. 2d).

Performing linear model analysis including both location and year of sampling as factors further highlighted these differences. Our results showed differences across years in the average starvation resistance values observed (significant year factor). Furthermore, the generation*year interaction term was also significant, indicating differences across years in the patterns of evolutionary response for this trait.

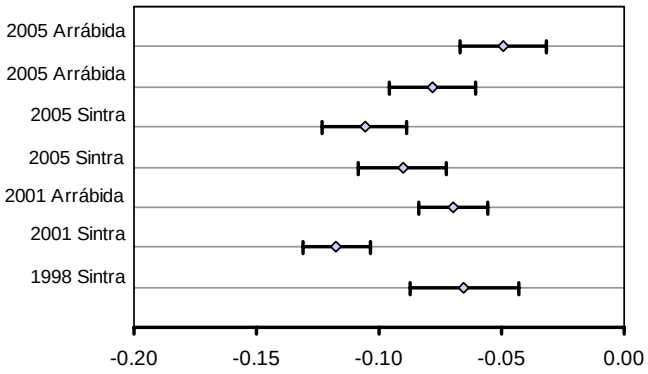

Figure 2A. A1R

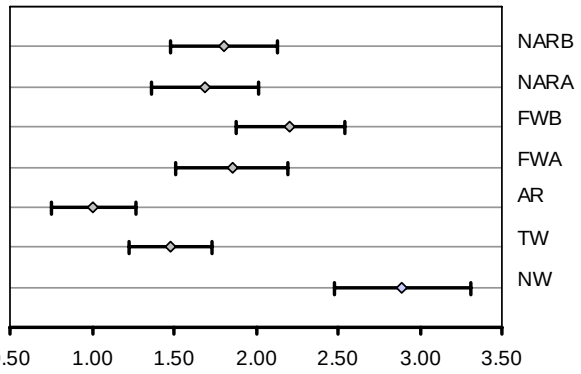

Figure 2B. F1-7

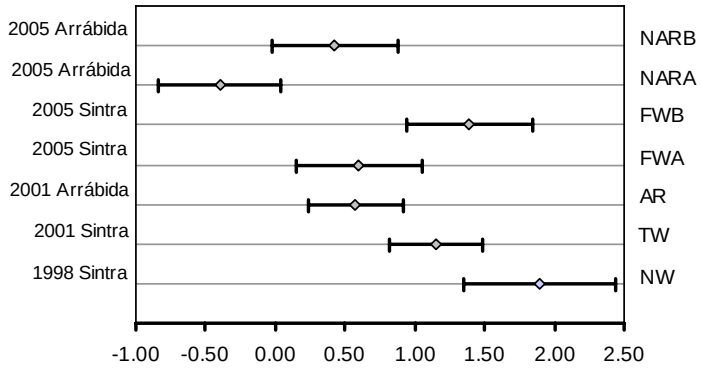

Figure 2C. F8-12

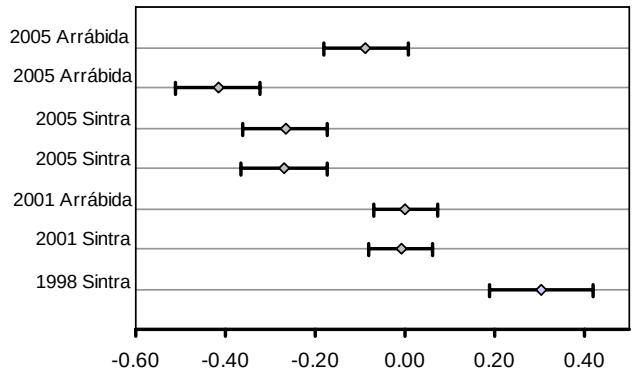

Figure 2D. RF

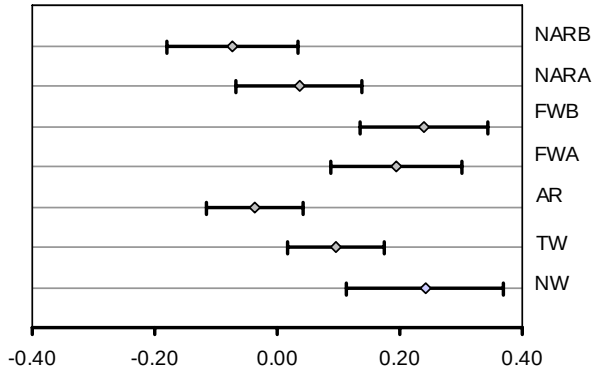

Figure 2E. RM

Figure 2. Evolutionary rates (slopes) for each of the seven foundations and five life history traits analysed. Standardized data was used in each analysis. (A) Age of first reproduction (A1R); (B) Early fecundity (F1-7); (C) Peak fecundity (F8-12); (D) Female starvation resistance (RF) and (E) Male starvation resistance (RM). The foundations shown are: NW (Sintra, 1998), TW (Sintra, 2001), AR (Arrábida, 2001), FWA and FWB (Sintra, 2005), NARA and NARB (Arrábida, 2005). For each trait and foundation the bar represents the standard error of the slope estimated from the model: trait ~ generation + foundation + generation*foundation (see Materials and Methods).

Male starvation resistance

A simple linear regression applied on the male starvation resistance data from all foundations indicated a significant generation effect, revealing a positive trend for this trait. Male starvation resistance also presented a significant generation*foundation interaction when comparing NW data with both AR and NARB, indicating different evolutionary dynamics between NW and these foundations (see Fig. 2e).

These differences between foundations were further investigated by performing linear model analysis including both location and year of sampling as factors. A significant year factor was obtained suggesting differences across years in the average starvation resistance values of this trait. Also, a significant generation*location effect was obtained, suggesting differences across locations in the patterns of evolutionary response for this trait.

An additional linear mixed effects model was also applied to the 2001 and 2005 data to test for differences between the evolutionary dynamics of geographically distinct populations between the two years analysed. No significant location*year or generation*location*year interaction term was found for any phenotypic trait measured. This indicates absence of temporal variation in the net differences of evolutionary response between the Sintra and Arrábida derived populations. Finally we performed a linear mixed effects model with the data of 2005, confirming the non-significance of sampling effects within locations, as obtained with our other, more conservative tests (first section).

As a final summary analysis, we also performed ANOVAs on the same body of data. For all traits there was a significant generation*foundation effect. The only exception was a non-significant interaction for the male starvation resistance data. We also obtained significant generation*location effect for age of first reproduction, peak fecundity and male starvation resistance and a significant generation*year effect for early fecundity and female starvation resistance (see also Fig. 2).

Discussion

Our results clearly support the importance of contingency in adaptive evolution. These contingent effects can apparently arise from both temporal and/or spatial components, changing among populations even within the same geographical region.

These evolutionary contingencies are expressed in differences in evolutionary rates across foundations. However, there is also a general pattern of adaptation across all foundations. Specifically, fecundity related traits show a clear improvement during laboratory culture for all foundations studied.

Our data also show that the relative importance of these contingencies varies depending on the specific character considered. The adaptive responses of traits such as age of first reproduction, peak fecundity and male starvation resistance differed significantly as a result of the geographical location of the sampling site. For early fecundity and female starvation resistance, the temporal component appeared to be more important in generating variation in the evolutionary responses of the populations. In the case of female starvation resistance, the different evolutionary dynamics across years correspond to an increasing trend in the 1998 foundation – see also Matos et al. 2002 – while across the other foundations this trend is negative or null (see Fig. 2). In fact, our previous studies have already suggested some lack of consistency in the results obtained for this trait across foundations (Simões et al. 2007). It is thus not possible to generalize or predict the evolutionary patterns during laboratory adaptation for this trait. This might partly explain disparities between laboratories with respect to the changes in starvation resistance during captivity (e.g. Hoffmann et al. 2001 cf. Griffiths et al. 2005).

One potential explanation for the higher temporal variability in the response of female starvation resistance and early fecundity may be temporal changes of selective pressures in the wild environment for these traits. This would likely lead to a significant differentiation between foundations, contributing to a temporal variation in the evolutionary response to the laboratory environment, even from foundations from the same natural site. It is also possible that these traits are more sensitive to genotype *vs.* environment interactions that cause different evolutionary rates among asynchronous foundations.

It is suggestive that two traits that are likely to share a fairly common genetic basis, specifically female and male starvation resistance, appear to be differently affected in their evolutionary patterns by contingent factors such as temporal or spatial variation in populations.

Another interesting result revealed by our meta-analysis is the consistent difference in the adaptive dynamics of geographically distinct populations across temporally separated foundations. In fact, differences in evolutionary response between Arrábida and Sintra populations remained more or less stable through the years sampled.

Variation in Adaptation during Laboratory Evolution

This study reveals significant variation in initial evolutionary responses among traits during the initial generations of adaptation to a new environment. The existence of such variation in responsiveness has been inferred before in our previous studies, which have separately treated temporal (e.g. Matos et al. 2002) and spatial (e.g. Simões et al. 2007) effects. The present study involves both further replication of that earlier work and an overall meta-analysis of the entire series of experiments, involving seven independent foundations stretching over almost ten years. As such, our analysis provides the most complete analysis of the adaptive evolution of a species in response to the same selective regime, when multiple foundations from wild populations at different locations and different times are employed.

The results of this study parallel those of other authors who started their laboratory evolution experiments from different source populations. For example, Cohan and Hoffmann (1986) found that *Drosophila melanogaster* populations derived from collections obtained from wild populations at very different latitudes along the west side North American coast showed different correlated responses to selection for knockdown resistance to ethanol. Teotónio and his collaborators (Teotónio and Rose 2000; Teotónio et al. 2002) performed a reverse evolution experiment, in which genetically differentiated populations were returned to their common ancestral environment and then allowed to evolve in parallel for 50 generations. They found significant heterogeneity among the evolutionary trajectories of these populations in the same environment. Teotónio et al. concluded that past selective history along with a variable relationship between life history characters and fitness were responsible for the

heterogeneity that they observed. Both of their studies indicated effects of genetic background on evolutionary response. In contrast, other experimental evolution studies in *Drosophila* have shown that the effects of history are transient (e.g. Joshi et al. 2003). It is possible that the differences that we observe in the initial stages of adaptation of our laboratory populations are reduced in the long term. Studies with more generations will clarify this issue.

The longstanding experimental evolution work of Lenski and colleagues using *E.coli* has also addressed the effects of historical contingencies in evolution (Travisano et al. 1995; Elena and Lenski 2003). In this particular experiment, *E.coli* lines that had previously evolved in glucose for 2,000 generations were placed in a maltose environment for another 1,000 generations. The evolutionary response in this new environment was measured in 36 *E.coli* populations – as a result of the foundation of 3 replicate populations from each of the 12 populations that previously evolved in glucose (see Travisano et al. 1995). The results showed that the replicate derivatives of these populations evolving in the new maltose environment achieved similar fitness levels despite prior history and/or subsequent chance events. On the other hand, chance and historical events were found to have a clear impact on traits less correlated with overall fitness (e.g. cell size), leading to differentiated response among populations in these particular traits (Travisano et al. 1995).

Thus, using very different methodologies, it is generally found that evolutionary contingencies tend to produce some degree of variability in the adaptive dynamics of laboratory evolution. It is important to note that this is variability in the evolution of characters that are demonstrably significant for the functional evolution of these populations, in most instances. Thus, it is well established that such *Drosophila* characters as early fecundity and starvation resistance are heavily implicated in the adaptation of diverse laboratory cultures from this genus (e.g. Rose et al. 2004), just as competitive fitness is definitively established as functionally important for the evolution of laboratory *E. coli* cultures (e.g. Elena and Lenski 2003; Lenski 2004). It is not as if the variation in evolutionary dynamics that we and others have adduced is variation in the evolution of neutral, functionally irrelevant, or otherwise minor characters. Rather, from the vantage point afforded by our studies and others like them, there is adaptively significant variation in the outcome of evolution as a result of initial differentiation.

What can we say about the wild populations that we sample and subject to laboratory evolution?

The present study is different from those of Teotónio and colleagues (e.g Teotónio and Rose 2000; Teotónio et al. 2002) or Lenski and colleagues (Travisano et al. 1995) in that the genetic differentiation that our experimental populations start with comes from nature.

Some authors have argued that the use of long-established laboratory populations limits inferences about evolutionary processes (Harshman and Hoffmann 2000; Linnen et al. 2001). This view assumes that experimental evolution studies aim at extrapolating values expressed by laboratory populations to what should be found in the wild. However, this is not necessarily the goal of such studies. Experimental evolution refers primarily to the test of predictions regarding evolutionary processes and the study of the evolutionary potential underlying responses to selective pressures. In our view, the laboratory environment is just a new environment featuring a set of new challenges to which populations may adapt (Matos et al. 2000b). Moreover, we propose that the laboratory environment can in fact be an ideal setting to address the potential for adaptive responses and to test general predictions concerning evolutionary patterns, such as convergence. The analysis of the laboratory adaptation process of recently wild-collected samples allows experimenters to study in detail the evolutionary response of populations with high starting genetic variability as well as to address issues such as the impact of different genetic backgrounds during evolution, or the repeatability of evolutionary patterns across temporally and spatially sampled populations, as we have here.

We do not propose that our particular findings allow us to infer specific features of the wild populations of *D. subobscura* that we sampled originally. However, our findings suggest that these populations may vary significantly within a relatively small geographical area over a relatively short period of time. It is of course possible that all the effects that we detect are merely due to sampling effects, and that there are no actual differences in the make-up of the populations that we have sampled repeatedly. However, we find this unlikely as a general explanation of the differences found, given the similarity in the two samples obtained from each location in 2005, particularly for age of first reproduction, early fecundity and male starvation resistance. Nevertheless, both peak fecundity and female starvation resistance results suggest that sampling

effects may have played some role in the different evolutionary dynamics among foundations (see Fig. 2), but only more data across locations and years would allow an accurate test of these effects.

Nonetheless, our long-term research program is perhaps the first in which it is possible to address the question of whether or not populations in the wild differ with respect to their capacity to respond to a novel selective regime. As such, it provides a powerful and novel window into the potential for adaptive evolution of populations in the wild, a window very different from that provided by the collection of data pertaining to standing genetic variation in the wild. The latter has been a traditional research topic within population genetics, from the pioneer studies of Dobzhansky and Lewontin (Dobzhansky 1937; Lewontin and Hubby 1966) to the recent attempts to detect selection in natural populations (see Ford 2002, for a review). It is also a different angle on this question from that afforded by studies of the phenotypics of selection in nature (e.g. Lande 1979; Lande and Arnold 1983; Arnold and Wade 1984a,b; Grant and Grant 1995; Reznick et al. 1997).

What we are suggesting is that the laboratory evolution of a sample collected from a wild population is a third kind of assay of the evolutionary state of that wild population. While any such laboratory evolution experiment is necessarily limited to the particular selection regime(s) chosen, it provides one of the most readily interpretable assays of the potential for adaptation of a population. As such, this particular type of assay should perhaps be more commonplace among the experimental designs used in evolutionary research.

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Chapter 5.

Divergent evolution of molecular markers during laboratory domestication in *Drosophila subobscura*

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Abstract

The impact of genetic drift in population divergence can be elucidated using replicated laboratory experiments. In the present study we applied microsatellite analyses to study the genetic variability and differentiation of laboratory populations derived from a common ancestral natural population after 49 generations in the laboratory environment. We found substantial genetic variability in all our populations. The decline of genetic diversity was mild (11 to 13%) and very similar in our long term established replicated populations, even though they were clearly adapting to the laboratory environment. Nevertheless, genetic drift led to significant genetic differentiation between them with an F_{st} of 0.104. Effective population sizes were clearly influenced by variation in reproductive success associated with the ongoing adaptive process occurring in these populations: estimates ranged between 17% and 47% of the registered census size, depending on the estimation method used.

The low decline in genetic variability throughout captivity, despite an adaptive event occurring in these laboratory populations, suggests that careful maintenance procedures can efficiently reduce the loss of genetic variability in captive populations, even without applying active management procedures with conservation purposes.

Keywords: Domestication; Genetic drift; Effective population size; Captivity; Microsatellites; Divergent evolution.

Introduction

Random genetic drift is a powerful mechanism producing evolutionary changes at the population level (Crow and Kimura, 1970). It is a stochastic process associated with sampling a finite number of gametes during reproduction of the individuals in a population, causing random allele frequency changes at each generation, at a rate that is dependent on the number of effective breeders (i.e. effective population size) (Falconer and Mackay, 1996). As a consequence of these sampling effects across generations, some alleles will eventually be lost while others may become fixed leading to loss of genetic variability within populations. Also, the fate of these alleles will vary among populations leading to progressive genetic differentiation between populations (Hartl and Clark, 1989).

The loss of genetic variability is particularly important in small and confined populations, because the effects of genetic drift are not counteracted by the input of new alleles. Several conservation studies have tried to deal with this issue, developing strategies to maximize the genetic diversity in captive populations (e.g. see Caballero and Toro, 2000; Frankham *et al*, 2002). These include, for example, procedures aiming to equalise family contributions (i.e. to increase the effective population size) or managing these contributions to minimize global coancestry between parents (when pedigree information is available). Another relevant procedure is the fragmentation of captive populations with periodical inter-cross between them, as a way to rescue genetic diversity, since different alleles may become differently fixed among populations (e.g. see Frankham *et al*, 2002).

Laboratory studies can be useful to analyse an evolutionary process in action and, through the study of the changes in the genetic composition of different populations in a temporal perspective, address the impact of genetic drift in both genetic diversity and population differentiation. Through previous knowledge about the history of the populations under analysis it is possible to disentangle the effects of different evolutionary mechanisms (genetic drift, founder effects, population size, selection, migration etc.), contrary to most studies in natural populations (Morgan *et al*, 2005; Chippindale, 2006).

Several recent empirical studies in laboratory populations have focused on how different population sizes affect the rates of genetic diversity decline (e.g. see Montgomery *et al,* 2000; England *et al,* 2003; Rodríguez-Ramilo *et al,* 2006).

Fewer studies in laboratory populations analyse the effects of genetic drift upon population differentiation as populations adapt to a common environment (but see Morgan *et al*, 2003). In spite of this lack, it is a known fact that genetic drift should be taken into account in experimental evolutionary studies, traduced in the need for having replicate populations in all studies (Rose *et al*, 1996). But how much genetic differentiation actually occurs between these populations? This is a relevant question both at a scientific and methodological level, and it is surprising that so few studies have addressed it, given the huge amount of biological material available to tackle the issue (e.g. for reviews of experimental evolution studies see Prasad and Joshi, 2003; Chippindale, 2006).

Analysis of laboratory-controlled populations of known history also allows estimating the effective population size (N_e), a key parameter in both population and quantitative genetics (Wang and Caballero, 1999; Wang, 2005). The effective population size determines the strength of stochastic events thus influencing decisively the action of random drift and all the genetic consequences associated with it (Falconer and Mackay, 1996; Frankham *et al*, 2002). Therefore, the maintenance of a high effective population size is a priority in conservation programmes of captive populations (Caballero and Toro, 2000; Frankham *et al,* 2002). Often there is no knowledge on the effective size of populations, and the use of census size as proxy may be quite misleading. Most studies have reported extremely low ratios of effective sizes relative to census sizes for both laboratory and natural populations (e.g. Briscoe *et al*, 1992; Frankham *et al*, 2002). This is due to the fact that N_e can be reduced by diverse aspects such as fluctuations in population size over generations, variation in family size, differences in sex ratio, mating system, selection, etc… (Frankham, 1995; Wang, 2005). Frankham (1995) reported an average of 0.11 for N_e/N ratios in natural populations, with a wide range of values (between 0 and 0.6), probably due both to the diverse influence of these factors and to lack of precision of the estimates, particularly in natural populations (see Frankham *et al*, 2002). Laboratory studies, given the possibility to determine both effective and census sizes more accurately relative to natural

populations, can be a valuable tool to empirically estimate N_e/N ratios and approach possible factors that affect them.

In the present study we analyse the genetic variability and differentiation of laboratory populations derived from a common ancestral natural population after prolonged evolution in the laboratory environment. Specifically, we characterize 10 microsatellite loci in three laboratory replicate populations of *Drosophila subobscura* after 49 generations of adaptation to the laboratory (NW populations). We compare the data obtained in these populations with another set of populations (TW populations) recently introduced in the same environment and collected from the same natural location (see Matos *et al,* 2004), using the same set of microsatellite loci. The TW populations are used as a measure of initial genetic variability close to laboratory foundation.

The specific aims of our study are: *i)* to analyse the genetic variability in populations evolving for 49 generations in a constant and common environment, as measured by molecular markers; *ii)* to study the genetic differentiation in these laboratory populations, after 48 generations of independent evolution from a common ancestral natural population; *iii)* to determine the effective population sizes as well as their ratio relative to census sizes in these laboratory adapting populations.

Materials and methods

Foundation and maintenance of the laboratory populations

In March 1998 the NW laboratory population of *Drosophila subobscura* was founded with 300 females collected from a pinewood near Sintra, Portugal. Collections of eggs laid by the first generation females were split, giving rise to three replicate populations from the second generation on, referred to as NW_{1-3} (Matos *et al,* 2002). In October 2001, an additional foundation (called TW) was carried out, from the same place, with 110 females. TW replicate populations were originated using the same procedure as described above for the NW populations. From the beginning all populations were maintained in similar conditions, e.g. discrete generations, reproduction at a young age (around 7 to 10 days after emergence)**,** control of temperature (18ºC) and densities with population sizes from 600 to 1 200 individuals (Matos *et al,* 2002; Simões *et al*, 2007).

Microsatellite analysis and genotyping methods

The three NW replicate populations (NW_{1-3}) were assayed at their 49th generation in the lab (corresponding to 48 generations of reproductive isolation). The three recently introduced TW replicate populations (TW_{1-3}) were analysed after three generations of laboratory culture (corresponding to first generation of reproductive isolation). Approximately 50 individuals were analysed for each one of the six populations mentioned above.

The ten microsatellite loci analysed in this study were: *dsub01*, *dsub02*, *dsub05*, *dsub10*, *dsub14*, *dsub19*, *dsub20*, *dsub21*, *dsub23* and *dsub27*. These markers were first identified and characterized by Pascual *et al.* (2000; 2001) and were chosen because of their polymorphism (after performing an initial survey with 30 individuals) and good coverage of the genome (chromosomal location given by Santos personal communication). *Dsub05*, *dsub19* and *dsub21* are X-linked and the others are autosomal.

Single fly genomic DNA was obtained using the extraction protocol described by Gloor *et al.* (1993). The forward primer of each locus was end-labelled with 6-Fam, Hex or Ned. All 10 loci were amplified using four multiplex PCR reactions: *dsub02+dsub05; dsub10+dsub14; dsub20+dsub21+dsub27; dsub01+dsub19+dsub23.* The amplification reactions were conducted for a total volume of 25 µl with 2.5 pmol of each primer, 3 µl dNTP´s (1mM), 2.5 µl 10 x buffer, 1 U *Taq* polymerase (Fermentas) and 1 µl of DNA.

All reactions were performed on an ABI GeneAmp PCR System 2700 machine using the following program: 5 min at 95º, then 30 cycles of 1 min at 95º, 1 min at 54º and 30 seconds at 72º followed by 5 min at 72º. The alleles were sized on an ABI PRISM 310 sequencer (Perkin-Elmer) using ABI GeneScan-500 ROX as an internal standard.

Microsatellite data analysis

The genetic variability in each locus and population was measured as the number of alleles, expected heterozygosity and observed heterozygosity using MSA software, version 2.32 (Dieringer and Schlötterer, 2003). To test for differences between NW and TW populations, Wilcoxon matched pairs tests were performed using as paired data the average estimate of each variability measurement, for each locus, in NW and TW

replicate populations. Tests for differences between all population pairs were also carried out on the same genetic parameters. These analyses were performed using Statistica 5.0.

Genetic differentiation between all population pairs F_{st} (Weir and Cockerham, 1984) was calculated using the MSA software. The significance of pairwise F_{st} values was tested by permuting genotypes among populations 10 000 times. To account for multiple testing, we used the sequential Bonferroni method whenever necessary (Sokal and Rohlf, 1995).

We tested the hypothesis that genetic differentiation, as measured by global F_{st} values was higher in the NW than in the TW set of populations using a permutation test (10 000 iterations) in the FSTAT software (Goudet, 2001).

A Bayesian clustering analysis (clustering of groups module) was also performed using BAPS 3.2 software (Corander *et al,* 2005). This program uses the allelic frequencies and pre-defined number of populations as random variables and generates a posterior likelihood of population structure (clustering).

A hierarchical analysis of molecular variance (AMOVA) was performed to analyse the distribution of the total genetic variance due to three different sources of variation: between groups (NW and TW), between populations/within groups and within populations. A locus by locus AMOVA was also performed to discriminate the partition of the total variance for each locus separately. These analyses were carried out using ARLEQUIN, version 2.000 (Schneider *et al,* 2000).

Effective population sizes (N_e) for each NW population were estimated from microsatellite data using three methodologies: the *F*-statistic method (Nei and Tajima, 1981; Waples, 1989), a pseudo-likelihood approach (Wang, 2001) and also the loss of heterozygosity method (see Crow and Kimura, 1970). Both the *F*-statistic method and the pseudo-likelihood approach use the changes in allele frequencies in temporal samples to estimate the average effective population size in the interval considered assuming genetic drift as the only factor causing those changes (Nei and Tajima, 1981; Waples, 1989; Williamson and Slatkin, 1999). N_e was calculated for each NW population assuming that TW populations represent, at an acceptable degree, the allele frequencies that NW populations had at their generation 3.

For the *F*-statistic method, N_e estimates for NW populations were calculated through the standardized variance of allele frequencies (F_c) between two samples: data

from generation 3 of TW_2 population and from generation 49 of each NW population. Analyses using TW_1 and TW_3 populations as the initial samples gave similar results as those using TW_2. F_c was calculated for each locus according to equation (15) in Nei and Tajima (1981). A weighted mean of F_c values across loci was used to estimate N_e according to equation (18) (sampling scheme II) in Nei and Tajima (1981). Confidence Intervals for F_c were computed with equation 16 from Waples (1989).

The effective population sizes of NW populations were also estimated with a pseudo-likelihood approach implemented in MLNE (Wang, 2001) using the same body of data mentioned above. All analyses were performed allowing a maximum N_e value of 1 000.

Finally the effective population sizes (N_e) for each NW population were also estimated from microsatellite data using the formula $H_t/H_0=(1-1/2N_e)^t$ (Crow and Kimura, 1970). The data used was the expected heterozygosity of NW populations at generation 49 (H_t) and the expected heterozygosity of TW populations at generation 3 (H_0). N_e was calculated substituting t by 46, the number of generations between the two sets of populations. For each NW population we estimated N_e using as initial heterozygosity the pooled estimate of the three TW populations.

Results

Genetic Variability in NW and TW populations

Table 1 summarizes the genetic variability detected in NW and TW laboratory populations. Overall, the data showed a high genetic variability in these laboratory populations. No significant differences in observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) and allele number (n_a) were obtained between NW replicate populations or between TW replicate populations (Wilcoxon matched pairs test, $p>0.05$). However, there were clear differences in genetic variability between NW and TW populations, being NW populations the less variable ones. The mean number of alleles per locus was significantly lower in NW populations when compared with TW (Wilcoxon matched pairs test; Z = 2.803, d.f.= 18, $p<0.006$). Expected heterozygosity and observed heterozygosity were also significantly lower in NW populations

(Wilcoxon matched pairs test; $Z = 2.803$, $p<0.006$; and $Z = 2.191$, $p<0.029$, respectively).

Table 1. Genetic variability in NW and TW laboratory populations.

Regime	Population	n	n_a	H_{obs}	H_{exp}
NW.....	NW_1	44.8	7.8	0.681	0.732
	NW_2	45.7	9.8	0.695	0.740
	NW_3	47.6	8.7	0.744	0.747
TW.....	TW_1	46.2	15.4	0.772	0.833
	TW_2	46.6	16.2	0.808	0.847
	TW_3	46.9	16.1	0.755	0.838

Note.- n - mean number of individuals per locus; n_a - mean allele number per locus; H_{obs} - mean observed heterozygosity; H_{exp} - mean expected heterozygosity.

Assuming that the pooled TW genetic variability reflects the initial NW variability we calculated a decline of genetic diversity during the 49 generations of laboratory evolution of about 12.8%, 11.9% and 11.0% for NW_1, NW_2 and NW_3, respectively.

Genetic Differentiation in NW and TW populations

Significant genetic differentiation was observed for all NW population pairs and also for all NW *vs.* TW pairwise comparisons (see Table 2). Interestingly, pairwise F_{st} values between NW population pairs were consistently higher than those obtained for NW *vs.* TW pairwise comparisons. On the other hand, pairwise F_{st} values between TW populations were much smaller, mostly showing non-significant genetic differentiation with the exception of TW_1 compared to TW_2. Similar results were obtained when all loci or only autosomal loci were considered (data not shown).

The global F_{st} value among NW populations was significantly higher than the global F_{st} obtained among TW populations, as assessed over 10 000 permutations (p= 0.048; with F_{st} NW = 0.104, CI=0.075; 0.134; and F_{st} TW = 0.004, CI=0.002; 0.006).

A Bayesian multilocus genotype data approach was also used to assess genetic differentiation between our laboratory populations, giving a four-cluster optimal

partition for our data, one cluster for each NW population and another cluster for all TW populations.

Table 2. Pairwise F_{st} (above diagonal) and *p*-values (below diagonal) between NW and TW populations.

	NW_1	NW_2	NW_3	TW_1	TW_2	TW_3
NW_1	-	0.104	0.132	0.076	0.084	0.081
NW_2	**	-	0.076	0.055	0.050	0.051
NW_3	**	**	-	0.062	0.062	0.058
TW_1	**	**	**	-	0.006	0.005
TW_2	**	**	**	*	-	0.004
TW_3	**	**	**	n.s.	n.s.	-

Note.- n.s. $p>0.05$; * $0.01<p<0.05$; ** $p<0.01$

A global AMOVA performed on the NW and TW groups of populations assigned the highest percentage of variation - 93.61% - to the within population (between individuals) level. The between groups (NW *vs.* TW) and the between populations / within groups source of variation explained 1.10% and 5.29% of the total variation, respectively. Only the between groups variance component was not significant as assessed by permutation.

Table 3 shows the percentage of variation attributed to each variance component for each locus independently. As expected by the results obtained from the global AMOVA, the V_c (variation within populations) component includes the majority of variation obtained. However, it is interesting to note that the percentage of variation associated with the V_b (between populations / within groups variance) component was higher for the microsatellite loci located in the A (sexual) chromosome. For the V_a (between groups) component, it is worth mentioning that the three loci with the higher percentage of variation (*dsub01*, *dsub02* and *dsub14*) are located in the same (O) chromosome.

Table 3. Percentage of variation of ANOVA variance components per locus.

Locus	Chrom.	% V_a	% V_b	% V_c
dsub05	A	1.58	9.04	89.38
dsub19	A	-0.06	6.46	93.60
dsub21	A	0.63	8.65	90.72
dsub23	J	0.83	4.35	94.82
dsub27	J	1.06	4.12	94.82
dsub10	U	-0.30	6.95	93.35
dsub20	E	0.88	2.32	96.80
dsub01	O	2.31	3.05	94.63
dsub02	O	2.05	3.58	94.38
dsub14	O	3.77	3.01	93.21

Note.- Chrom. - Chromosome; location assessed by FISH (Santos personal communication);

V_a - between groups variance component (NW *vs.* TW);

V_b - between population/within groups variance component;

V_c - within population variance component.

Demographic History of NW populations

Table 4 shows the effective population size (N_e) and N_e/N ratios for NW populations using different estimators, with N being the average census population size estimated per generation. Based on the loss of genetic diversity through time, all NW populations showed quite similar effective population sizes, with a ratio between effective population size and census size ranging between 0.173 and 0.215.

The effective population sizes estimated according to Wang (2001) were quite similar to those obtained through the loss of genetic diversity, except for the NW_2 population with a higher effective population size. As for the *F*-statistic estimate (Nei and Tajima, 1981), the N_e values were higher than those obtained with the previous approaches, although differences were only statistically significant for the NW_3 population. As a result, the ratio between effective population size and census size was also higher for this method (see Table 4).

Table 4. Estimates of effective population size (N_e) and N_e/N ratios for NW populations.

Population	N_{e*}	N_{e**} (95% CI)	N_{e***} (95% CI)	N	N_{e*}/N	N_{e**}/N	N_{e***}/N
NW_1	168	151.8 (122.2-189.4)	282.9 (177.9-371.1)	968.8	0.173	0.157	0.292
NW_2	182	280.8 (222.7-356.1)	441.4 (324.4-597.2)	947.5	0.192	0.296	0.466
NW_3	197	210.7 (167.4-265.6)	366.0 (271.6-488.2)	916.7	0.215	0.230	0.399

Note.- N_e* - effective population size estimated by the loss of heterozygosity method; N_e** - effective population size estimated according to Wang (2001); N_e*** - effective population size estimated according to Nei and Tajima (1981); N - census population size.

It is worth noting that the NW_1 population had the lowest N_e and N_e/N values (see Table 4) for all estimates. It was also the population with the lowest genetic diversity values (see Table 1).

Discussion

Microsatellite Variability in Laboratory Populations

Our laboratory populations showed an overall high genetic variability in the 10 microsatellite loci analysed in this study (see Table 1). Nevertheless, the genetic variability of our longer established populations (NW) was significantly lower than the recently established populations (TW). This difference is most likely due to genetic drift (see next section).

The results obtained for our recently established TW populations resemble those of the non-bottlenecked European populations reported by Pascual *et al.* (2001), with similar genetic variability both in terms of expected heterozygosity (0.840 and 0.874, respectively) and average allele number (16.0 and 15.6, respectively). This suggests that the foundation process and the three generations in laboratory culture produced little impact in the genetic diversity of TW populations. Our *a priori* assumption that TW genetic variability reflects that presented by NW populations in the first generations of laboratory culture seems thus highly probable, an expectation reinforced by the fact that these populations were founded from the same natural location.

As expected given the higher number of generations in the laboratory, NW populations had a smaller genetic diversity than TW populations, due to the progressive loss of genetic variability through genetic drift.

It is interesting to note that NW expected heterozygosities (0.740) are closer to those obtained for American natural populations (0.727), which present lower genetic variability than European populations as a result of a strong founder effect associated with the colonization of America (Pascual *et al,* 2007). Nevertheless, NW populations present a considerably higher average allele number than the American populations (8.8 and 5.5, respectively). This is probably due to the mild bottleneck in our populations as a result of laboratory culture in contrast to the severe bottleneck associated to the colonization event (Pascual *et al,* 2007). The discrepancy encountered between

heterozygosity and allele number is an expected outcome since strong bottleneck events reduce allele number faster than expected heterozygosity (Nei *et al,* 1975; England *et al,* 2003).

The genetic variability of our long captive NW populations is higher than that reported in a similar work by Rodríguez-Ramilo *et al.* (2006) dealing with *Drosophila melanogaster* populations after 38 generations in the laboratory (H_{exp}=0.470). The initial expected heterozygosity was also lower in those populations, with an average value of 0.56 contrasting with an average of 0.84 in our TW populations. These differences might be caused by the fact that our populations were derived from a natural population of the ancestral area of the species while *D. melanogaster* populations in that study were derived from a colonized area.

The percentage of decline in genetic diversity was very similar between all NW populations (11 - 13% from the initial diversity). This corresponds to an average decline in genetic diversity of 0.003 per generation in our populations. This decline is considerably higher than the predicted decline of 0.0005 per generation considering N_e=N=950, which suggests a considerable difference between the census and effective population size on these populations (see below). This is likely due to variance in reproductive success that contributes to a decrease in the effective number of breeders (Falconer and Mackay, 1996). Indeed, our populations presented a clear adaptive process to captive conditions in this period (Matos *et al,* 2002).

The experimental populations of Rodriguez-Ramilo *et al.* (2006) maintained with an N of 100 presented a larger decline of genetic diversity (16%) in spite of the shorter period of time elapsed (38 generations). This corresponds to an observed decline in genetic variability of 0.005 per generation. Surprisingly, a similar decline is expected considering N_e =100, that is, if the census size equals the effective population size. This suggests absence of adaptation to the new captive conditions, which seems to be the case since global fitness did not improve across generations. This is somewhat unexpected given the diverse examples of adaptation to the laboratory (e.g. Matos *et al*, 2002; Gilligan and Frankham, 2003; Simões *et al*, 2007).

Loss of genetic variability and adaptation to captivity are two major problems in *ex-situ* conservation programmes (Frankham *et al,* 2002). In these programmes, maintenance of captive populations focus on maximizing effective population sizes

while, at the same time, reducing the effects of adaptation to non-natural conditions (see Caballero and Toro, 2000; Frankham *et al,* 2002; Rodriguez-Ramilo *et al,* 2006). The most common strategy to satisfy both requirements is to manage these populations by equalising parental contributions, i.e. where each individual contributes equally to the next generation (e.g. see Wang, 1997). However, few studies have compared the performance of long-term captive populations under managed *vs.* non managed conditions. One such study was that by Rodríguez-Ramilo *et al.* (2006).

In managed *Drosophila melanogaster* populations with an N_e of approximately 200, Rodriguez-Ramilo *et al.* (2006) found a rate of heterozygosity loss per generation of 0.0009, considerably lower than the decline in non-managed populations (also lower than the expected - 0.0025 - rate of decline for this N_e). England *et al*. (2003), analysing managed populations with a N_e of 100 also observed a decline in genetic diversity, with a rate of 0.007 per generation. Our data lies thus between those of Rodriguez-Ramilo *et al.* and England *et al.* for managed populations. Though our populations were non-managed and clearly adapting to the laboratory conditions, the decline of heterozygosity was low, probably due to the high population census sizes (of around 950) in our populations. Furthermore, the relatively modest decline in genetic heterozygosity observed in our NW populations, even after 49 generations of adaptation to a confined environment, suggests that no major bottlenecks occurred during this period. A careful maintenance procedure is critical to avoid severe drops in the genetic diversity of captive populations. In the present work these procedures included moderately high populations sizes and precautions to prevent non-random mating (e.g. careful randomisation of individuals in vials after emergence), although not involving direct management of individual contributions to the next generation.

There is a legitimate concern that adaptation to captivity may entail difficulties when populations are re-introduced in their natural environment (e.g. see Woodworth *et al*, 2002). However, strategies minimizing adaptation to captivity will imply a considerable reduction of selective forces that, in the long term will likely cause an increase in frequency of deleterious alleles (i.e. higher genetic load) leading to depression of overall fitness of these captive populations (Bryant and Reed, 1999). In this context non-management maintenance procedure may prove valuable particularly

for acceptably large populations experiencing prolonged captivity. Nevertheless, the best long-term strategy may vary from case to case.

Assessing the role of drift in causing genetic differentiation between populations

We presented evidence supporting a clear role of genetic drift in promoting genetic differentiation during the 49 generations of laboratory evolution in our laboratory populations. As expected, genetic drift effects lead to highly significant genetic differentiation between NW populations during evolution in the laboratory environment, with a global F_{st} of 0.104. This differentiation is explained by fluctuations of allele frequencies and the differential loss of alleles in NW populations, leading to a different subset of alleles in each NW population. The lower F_{st} values observed between NW and TW populations may be caused by the inverse relationship reported between locus polymorphism and F_{st} values (Carreras-Carbonell *et al*, 2006) as well as by the larger number of alleles present in TW populations including those also found in NW populations.

Few studies have analysed the genetic differentiation between laboratory populations evolving under a common regime. Morgan *et al*. (2003) analysed the dynamics of molecular markers in several laboratory lines of house mice under artificial selection for locomotor behavior. The hierarchical design used included two different selection treatments (selection groups *vs.* control) and four lines within each group. Significant genetic differentiation between lines (within a selection group) was observed after 14 generations, with an average value of 0.149, using microsatellite data. This value is of the same order of magnitude as those presented here for our NW populations (F_{st}=0.104). Given the discrepancy in the number of generations analysed between the two studies, a higher divergence between lines could be expected in our study. However, the higher effective population sizes in our populations relative to the house mice populations analysed by Morgan *et al*. (N_e=20) is likely to have counteracted the effect of the generation interval.

Morgan *et al.* (2003) also found no differentiation between selection groups. Overall, the authors concluded that genetic drift was the mechanism responsible for the divergence patterns observed between selection groups and between lines within selection groups.

Our data is comparable to that of Morgan *et al.* (2003). We also obtained no significant differentiation between NW and TW groups of populations. The absence of differentiation between the NW and TW groups is rather interesting since it suggests general neutral evolution of microsatellite markers in NW populations despite almost 50 generations of laboratory evolution and adaptation to captive conditions.

However, the impact of genetic drift in causing population differentiation can also differ between loci. Locus by locus AMOVA presented higher genetic differences between populations/within groups for all loci located in the A chromosome. The higher effect of genetic drift due to the lower effective population size of this (sexual) chromosome is likely to account for this result. On the other hand, higher percentage of variation between groups was observed in the three microsatellite loci localized in the O chromosome. This suggests that genes involved in laboratory adaptation may be in linkage disequilibrium with microsatellite loci in that chromosome. Studying the dynamics of the same laboratory populations through time is necessary in order to ascertain the validity of these hypotheses.

Inferring Demographic History through temporal changes in genetic parameters

The estimated N_e values for NW populations using different methods (loss of heterozygosity – see Crow and Kimura, 1970; temporal method - see Nei and Tajima, 1981; Wang, 2001) show an effective population size (N_e) in our populations of around 17 to 47% of the actual census population size (N), depending on the estimation method used (see Table 4). Since no strong fluctuation of the census size was registered in our NW populations (data not shown), it is likely that the low effective size relative to the census size is a consequence of the high heterogeneity in the parental contributions to the next generation (variance in family sizes). In fact, previous studies of laboratory adaptation support an unequal contribution of genotypes for the next generation, as indicated by a clear selective response in several life-history traits (Matos *et al,* 2002; Gilligan and Frankham, 2003; Simões *et al,* 2007). Nevertheless, the N_e/N values that we found are in general higher than previously reported for either captive or wild populations (Briscoe *et al,* 1992; Frankham *et al,* 2002). Specifically, Briscoe *et al.* (1992) reported N_e/N ratios between 0.4 to 4% using the loss of heterozygosity method in captive *Drosophila melanogaster* populations. As stated above, the laboratory

conditions under which our populations are maintained may allow retaining larger effective population sizes and therefore higher variability levels.

N_e estimates based on the pseudo-likelihood method described in Wang (2001) and observed N_e values based on the decline of expected heterozygosity were fairly close (see Table 4). On the other hand, N_e estimates based on the method of Nei and Tajima (1981) were higher. These higher estimates are in agreement with empirical tests suggesting that likelihood based approaches yield more reliable predictions of effective population size than F-statistic estimators, particularly with a high number of rare alleles (Wang, 2001, 2005).

General Conclusions

Genetic drift did not lead to a major loss of genetic variability within populations after 49 generations in a confined environment. Nevertheless, its action is clearly manifested in the significant genetic divergence between populations. Thus, genetic drift has acted through changes in allele frequencies among populations without causing major changes in the degree of genetic diversity in each population.

The low decline in genetic variability of our populations throughout captivity, even though they were clearly adapting to the new environment, suggests that careful maintenance procedures (e.g. high and fairly constant census sizes, randomisation to avoid non-random mating) can efficiently reduce the loss of genetic variability in captive populations, without necessarily applying active management procedures with conservation purposes. These results suggest that, for populations that produce a huge amount of offspring each generation, the best long term strategy may be to allow these populations to evolve freely in captive conditions as similar as possible to their natural environment.

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Chapter 6.

Evolutionary dynamics of molecular markers during local adaptation:

a case study in *Drosophila subobscura*

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Abstract

Background

Natural selection and genetic drift are major forces responsible for temporal genetic changes in populations. Furthermore, these evolutionary forces may interact with each other. Here we study the impact of an ongoing adaptive process at the molecular genetic level by analyzing the temporal genetic changes throughout 40 generations of adaptation to a common laboratory environment. Specifically, genetic variability, population differentiation and demographic structure are compared in two replicated sets of *Drosophila subobscura* populations recently sampled from different wild sources.

Results

We found evidence for a decline in genetic variability through time, along with an increase in genetic differentiation between all populations studied. The observed decline in genetic variability was higher during the first 14 generations of laboratory adaptation. The two sets of replicated populations showed overall similarity in variability patterns. Our results also revealed changing demographic structure of the populations during laboratory evolution, with lower effective population sizes in the early phase of the adaptive process. One of the ten microsatellites analyzed showed a clearly distinct temporal pattern of allele frequency change, suggesting the occurrence of a selective sweep affecting that particular locus. Globally, our temporal data indicated higher Q_{st} than F_{st} values.

Conclusions

Genetic drift was responsible for most of the divergence and loss of variability between and within replicates, with most changes occurring during the first generations of laboratory adaptation. We found overall similarity of evolutionary dynamics at the molecular level in our laboratory populations, despite distinct genetic backgrounds and some differences in phenotypic evolution. Thus, inferences of potential for adaptation based on microsatellite variability patterns among populations should be made with caution.

Background

Evolution in a novel environment involves a complex array of processes that lead to both genetic and phenotypic changes. The extent of these changes vary as a function of several forces, such as the selective pressures imposed and the magnitude of genetic drift, as well as the genetic background and prior evolutionary history of the populations concerned. Natural selection is evidently an important evolutionary process affecting differentiation between populations. Different selective regimes foster evolutionary divergence, while common novel selective forces are expected to lead to convergence [1]. Nevertheless, there is no certainty about the evolutionary outcome when multiple selectively differentiated populations adapt to the same environment (e.g., [2]).

An important evolutionary factor leading to differences among populations is genetic drift, particularly in populations with low effective size [3]. Moreover, natural selection and drift may interact, leading to disparate evolutionary outcomes among populations sharing a common environment (see [4,5]). Genetic drift can promote the loss of different alleles among isolated populations, potentially affecting the evolutionary response of selected traits that are influenced by such alleles. In addition, directional selection can reduce effective population size, enhancing the impact of genetic drift on genetic variability within populations and differentiation among them (see [6]).

Experimental evolution can help address these issues through the use of controlled selection regimes, controlled population sizes, and replication, both simultaneous and sequential [7]. In particular, the study of the evolution of laboratory populations since their foundation from the wild allows us to study the effects of population of origin, demographic structure, and the absence of gene flow on the process of evolutionary domestication. This experimental paradigm has the additional interest arising from the common pattern of larger population sizes in the population(s) of origin, and thus typically high initial genetic variability. All of this makes the study of adaptation to the laboratory well-suited to the analysis of the roles of selection, genetic drift, and their interaction during evolution in a novel environment [8]. In this setting, the evolutionary dynamics of molecular markers during laboratory adaptation offers the possibility of clarifying the impact of an ongoing adaptive event at the

molecular genetic level. Few studies have collected such information in an experimental evolution framework (but see [9,10]).

The joint study of evolutionary changes in selectively-important quantitative traits, such as those that define life histories, and highly polymorphic molecular markers, such as microsatellites, may allow some disentanglement of the effects of natural selection and genetic drift in population differentiation [11,12]. Combining these two types of data allows the comparison of differentiation among molecular markers, as measured for example by F_{st} [13], with the differentiation of such quantitative-character analogues as Q_{st} [14]. In this context, estimates of the F_{st} parameter across arbitrary molecular markers arguably might reflect the divergence between populations due to neutral causes. If F_{st} equals Q_{st} then it is conceivable that differentiation in quantitative traits might be explained by genetic drift alone. On the other hand, if Q_{st} exceed F_{st} values, directional natural selection might be promoting differentiation of quantitative traits more than expected by genetic drift alone [11,12].

Several recent studies have used this approach to address the evolutionary history of natural populations [15,16,17,18,19]. Nevertheless, the accuracy of this approach has seldom been tested. In a recent study [20], Q_{st} *vs.* F_{st} comparisons were evaluated using laboratory populations of house mice with known histories of evolutionary divergence. These comparisons were found to report the correct evolutionary inference at each level in the population hierarchy. Here we use a similar approach with our laboratory adapting populations, confronting temporal changes in life history traits with those of microsatellite loci. Our prior studies indicated clear, phenotypic, laboratory adaptation in these populations [8]. We can thus test the expectation of $Q_{st} > F_{st}$ in these populations.

Multilocus screens have been used to identify regions of the genome that have undergone positive selection (e.g., [21,22]). These tests rely on the assumption that regions subjected to positive selection will deviate from the neutral pattern that is assumed to be present in the remainder of the genome. Microsatellite loci, given their high polymorphism, wide distribution and abundance in eukaryotic genomes, are particularly suited for these screens [22,23]. Although microsatellite markers are often assumed to be neutral in themselves (see [24]), they can be affected by selective forces if linkage disequilibrium with a selected locus occurs, an effect known as “hitchhiking” [25]. The spread of a beneficial allele in an adapting population is expected to cause a

reduction of variability in the selected locus and its flanking regions [21,26,27] - a "selective sweep". Studying polymorphic microsatellite markers in populations adapting to a new environment should help evaluate their ability to detect loci that deviate from neutral expectations and, at the same time, might reveal regions of the genome implicated in adaptive processes [21].

Here we present a detailed study of the temporal changes of molecular markers in two sets of replicated populations of *Drosophila subobscura,* as they underwent adaptation to a common laboratory environment. The study of the evolution of molecular markers in these populations is an excellent opportunity to address the relationship between the evolutionary dynamics of presumptively neutral markers and the evolutionary dynamics of life-history traits that are known to be involved in a process of adaptation.

Results

AR and TW genetic variability

Both AR and TW populations showed high initial genetic variability, as measured at the third generation of laboratory adaptation (see Table 1).

However, significant differences were observed between loci for both allele number and expected heterozygosity in each generation, with microsatellite locus *dsub14* presenting the lowest mean allele number and the lowest expected heterozygosity in all generations analyzed [see Additional files 1, 2 and 3: Genetic Variability of AR and TW populations at generation 3, 14 and 40, respectively]. *Post hoc* Scheffé tests on expected heterozygosity also showed significant differences between *dsub14* and all other loci (data not shown).

There was a significant decline in allele number across generations in both sets of populations (AR: $F = 27.874$, $p = 0.000$; TW: $F = 20.956$, $p = 0.000$; bifactorial mixed ANOVA). With respect to expected heterozygosity, TW populations underwent a significant decline in expected heterozygosity across generations ($F = 4.527$, $p = 0.026$). AR populations, on the other hand, did not show a significant decline across generations for expected heterozygosity ($F = 1.748$, $p = 0.202$).

Table 1. Genetic Variability in AR and TW laboratory populations.

Regime	Population	Generation	n^a	$n_A{}^b$	$H_{exp}{}^c$
AR.....	AR_1	3	29.3	12.6	0.816
	AR_2	3	28.9	13.2	0.831
	AR_3	3	29.5	13.7	0.829
	AR_1	14	29.5	9.9	0.804
	AR_2	14	29.5	10.2	0.807
	AR_3	14	28.8	11.0	0.812
	AR_1	40	29.4	8.9	0.779
	AR_2	40	29.6	8.1	0.773
	AR_3	40	29.1	8.9	0.790
TW.....	TW_1	3	28.5	13.6	0.835
	TW_2	3	28.8	14.0	0.838
	TW_3	3	29.4	14.0	0.828
	TW_1	14	29.2	10.1	0.791
	TW_2	14	29.9	10.6	0.812
	TW_3	14	29.5	10.2	0.760
	TW_1	40	29.3	9.0	0.738
	TW_2	40	29.8	9.5	0.753
	TW_3	40	29.9	8.2	0.764

Note.- [a] Mean number of individuals per locus; [b] Mean allele number per locus; [c] Expected mean heterozygosity.

Trifactorial mixed ANOVAs were performed to test for differences in the rate of genetic variability decline between *groups* (AR and TW), *periods* (G3-G14 and G14-G40), and *loci* (see Table 3). The arcsine transformation was applied to the expected heterozygosity values (ratios between generations), to meet the assumption of normality. To allow this transformation, heterozygosity ratios higher than 1 were rounded to unity. This happened mostly for microsatellite *dsub14* due to a temporal increase in heterozygosity in this particular locus [see Additional files 1 and 2]. The rate of decline in genetic variability was significantly different between periods, being higher in the first period (generations 3-14) for both average allele number per locus and expected heterozygosity. Significant differences in the rate of decline of genetic variation were also found among loci. However, this rate of decline did not differ between groups (see Table 3).

Table 2. ANOVA differences in allele number and heterozygosity between AR and TW groups.

Generation	*Allele Number*		*Heterozygosity*	
	F	*p-value*	*F*	*p-value*
3	4.205	0.071	0.885	0.372
14	0.004	0.954	0.801	0.394
40	0.336	0.577	1.458	0.258

Note.- Tests for comparisons between TW and AR were bifactorial mixed ANOVAs with group (AR and TW) and locus as factors.

Table 3. ANOVA differences in the rate of variability decline between groups, periods and loci.

	Allele Number		*Heterozygosity*	
Factor	*F*	*p-value*	*F*	*p-value*
Group	1.038	0.335	2.392	0.156
Period	14.371	0.004	9.270	0.014
Locus	2.150	0.034	2.741	0.008
Group*Period	1.636	0.233	0.222	0.649
Group*Locus	0.938	0.497	1.657	0.114
Period*Locus	3.239	0.002	1.334	0.233
Group*Period*Locus	1.017	0.434	1.947	0.057

Note.- A trifactorial mixed model was applied with group (AR and TW) and period (G14/G3 and G40/G14) as fixed factors and locus as random factor.

AR and TW genetic differentiation

AR and TW populations already differed significantly at generation 3 (average F_{st}=0.015 of the three pairwise comparisons between same-numbered AR and TW populations, e.g. AR_1 *vs.* TW_1) [see Additional file 4: Pairwise F_{st} comparisons between AR and TW populations]. This differentiation between AR and TW populations increased through time (average F_{st}=0.071 at generation 14; average F_{st}=0.104 at generation 40). This increase was significant between generations 3 and 14 (*t*-test p=0.006) but not between generations 14 and 40 (p=0.102) [see Additional file 4].

Genetic differentiation within each set of populations (AR and TW) was also analyzed by pairwise comparisons between replicate populations in each generation [see Additional file 5: Pairwise F_{st} comparisons within and across laboratory generations]. No within-set initial genetic differentiation (at generation 3) was obtained for either AR (F_{st} =0.004) or TW (F_{st} =0.001) sets of populations. On the other hand, all populations within each set showed significant differentiation at generations 14 (F_{st} AR: 0.027; TW: 0.038) and 40 (F_{st} AR: 0.065; TW: 0.078). In fact, permutation tests indicated that genetic differentiation increased significantly across generations in both sets of populations. In each generation, genetic differentiation was not significantly different between TW and AR.

Molecular Analyses of Variance (AMOVA) was performed to further investigate the genetic differentiation between our AR and TW groups in each generation. At generation 3, both among-groups (V_a) and within-populations (V_c) variance components were significantly different from zero (1.32% and 98.39% of total variation, respectively), while the among-populations/within-groups variance component (V_b) was not significant (explaining only 0.28% of total variation). This indicates significant initial variance in allelic frequencies between AR and TW populations for these molecular markers. At both generations 14 and 40, all variance components were significantly different from zero, with an increase in both V_a and V_b components relative to generation 3 (4.10% and 4.03%, for V_a; 3.12% and 6.91%, for V_b, at generations 14 and 40, respectively).

An AMOVA over the entire evolutionary process was also performed for each set of populations to compare generations 3 and 40 of laboratory adaptation (specifically AR G3 *vs.* AR G40 and TW G3 *vs.* TW G40). The results obtained for the AR and TW populations were quite similar, with all three variance components being significant according to permutation tests. The highest percentage of variation was assigned to the V_c within-populations component (95.28% in AR; 94.43% in TW), with both among-generations variance component (V_a) and the among-populations/within-generations variance component (V_b) explaining a small percentage of total variation (1.26% and 3.46% for AR; 1.55% and 4.02% for TW, respectively).

AR and TW effective population sizes

Table 4 presents N_e estimates for both AR and TW populations during the different periods of laboratory adaptation analysed: the first period (generations 3 to 14), the second period (generations 14 to 40) and also during the overall period (generations 3 to 40) using both a pseudo-likelihood approach and the loss of heterozygosity method. N_e values were estimated excluding microsatellite locus *dsub14* from the data, given its extremely low diversity and its increase in heterozygosity between generations 3 and 14. Furthermore, the disparity between *dsub14* and all other microsatellite loci may be due to non-neutrality at this locus, an assumption of all models estimating N_e. The case of this particular locus will be addressed further below.

Using both methods, effective population size estimates for the first period of laboratory adaptation were significantly lower than those obtained for the second period for both TW and AR sets of populations (*t*-tests using as data points the $\sqrt{N_e}$ estimates of the three replicate populations; $p<0.05$ for all estimates; see Table 4).

AR populations presented a significantly higher N_e than TW populations between generations 3 to 14, according to the loss of heterozygosity method (AR N_e value=125.67; TW N_e value=71.00; *t*-test; $p=0.04$). Nevertheless, the AR and TW N_e estimates obtained using the pseudo-likelihood method for this first period did not differ significantly (*t*-tests; $p>0.1$; see Table 4). In contrast, all effective population sizes estimates between generations 14 and 40 for both AR and TW sets of populations were not significantly different (*t*-tests; $p>0.1$; see Table 4).

AR and TW effective population sizes were also not significantly different when all 40 generations of laboratory adaptation were considered, regardless of the estimation method used. N_e/N ratios ranged between 12.7 to 28.1% in TW populations and between 19.6 to 31.2% in AR populations.

Table 4. Estimates of effective population size (N_e) for AR and TW populations.

	AR_1	AR_2	AR_3	TW_1	TW_2	TW_3
Generations 3 to 14						
N_e (pseudo-likelihood)	101.52	109.96	227.36	122.86	134.01	115.85
CI (95%)	(74.51-144.62)	(80.41-156.67)	(144.62-419.8)	(88.14-180.43)	(96.61-196.83)	(84.87-165.25)
N_e (Ht/Ho)	116.80	112.78	146.88	69.33	95.13	49.02
N (census)	841.67	800.00	820.83	816.67	895.83	816.67
Generations 14 to 40						
N_e (pseudo-likelihood)	304.42	268.77	395.01	274.10	313.29	389.06
CI (95%)	(209.72-469.60)	(186.20-411.28)	(255.97-677.39)	(209.88-364.82)	(235.63-425.67)	(275.45-573.51)
N_e (Ht/Ho)	282.10	203.60	437.50	160.70	257.50	-
N (census)	927.78	866.67	875.93	963.33	951.85	965.19
Generations 3 to 40						
N_e (pseudo-likelihood)	274.65	165.83	253.08	230.36	263.19	190.98
CI (95%)	(201.40-384.17)	(127.24-218.88)	(186.01-351.29)	(174.43-309.09)	(198.19-357.58)	(143.87-258.34)
N_e (Ht/Ho)	196.87	163.64	271.90	117.71	170.64	170.83
N (census)	897.30	836.49	871.62	924.59	936.49	916.49

Testing for positive selection during laboratory adaptation

Heterozygosity ratios (Ln RH ratios) were calculated for both sets of populations by comparing data between generations 3 and 14 as well as between generations 14 and 40. When comparing generations 3 and 14, Ln RH values were significantly different between loci, both in TW and AR populations (one-way ANOVA; $p<0.001$). Ln RH values for locus *dsub14* were significantly different from those obtained for all other loci in all six populations (*post hoc* Scheffé test; $p<0.0001$ for all comparisons) as a result of the increase in heterozygosity at this locus. Ln RH values between all other pairs of loci were not significantly different ($p>0.05$ for all comparisons). Also, standardized Ln RH values for microsatellite locus *dsub14* fall outside the 95% confidence interval of the standard normal distribution for all replicates (see Fig. 1). The pattern observed in locus *dsub14* was due to the increase in frequency of the same allele (120 bp) in all TW populations and the AR_3 population, while a different allele (with 116 bp) increased in frequency among both AR_1 and AR_2 populations. In TW populations, the allele that increased in frequency (120 bp) rose from an average initial frequency of 11.5% at generation 3 to 31.6% at generation 14. In the AR_3 population, the120 bp allele increased from 5% to 19.2% while the 116 bp allele increased in AR_1 and AR_2 populations from an average frequency of 5.2% to 15.5%.

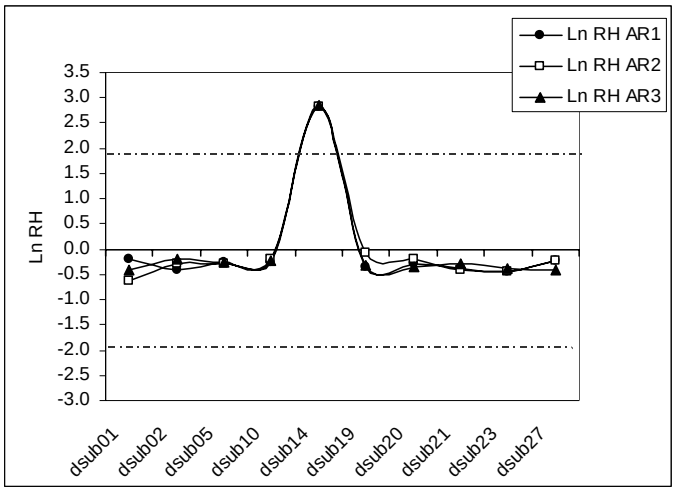

Figure 1A

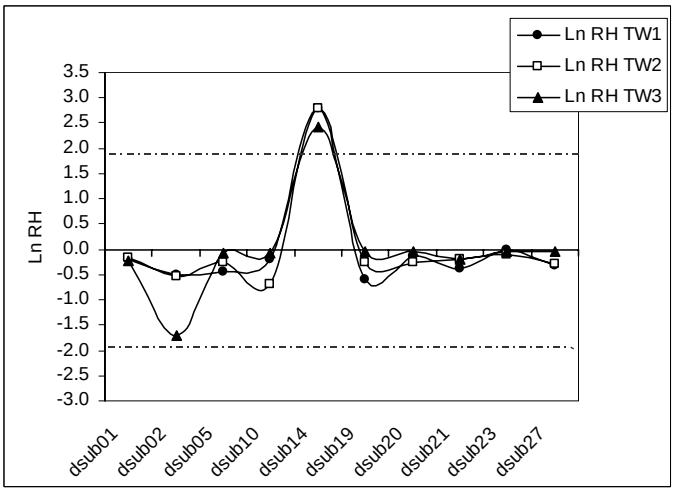

Figure 1B

Figure 1. Standardized Heterozygosity ratios (Ln RH) between generations 3 and 14 for AR (Fig. 1A) and TW (Fig. 1B) populations. Dashed lines represent the 95% confidence interval of the standardized normal distribution.

Between generations 14 and 40, Ln RH ratios for the AR populations were similar to those mentioned above, again with only locus *dsub14* significantly differing from all other loci (*post hoc* Scheffé test; $p<0.05$ for all comparisons). This was also observed for each replicate population by analysing the standardized Ln RH values (see Fig. 2). During this second period of laboratory evolution, the frequencies of the potentially selected allele in locus *dsub14* continued to rise in AR_1 and AR_2 populations (with the 116 bp allele reaching a frequency of 27.8 and 41.4% at generation 40, respectively). Nevertheless, in the AR_3 population the allele that had previously increased in frequency (120bp) slightly decreased (from 19.2% to 13.5%), being the high Ln RH ratio due to the increase in frequency of other alleles. For the TW populations, no significant differences between loci were detected with the general ANOVA or the Scheffé test. However, the analysis of the standardized Ln RH values for each TW replicate population showed some significant results, though they were not consistent among replicates. Specifically, locus *dsub14* showed a significant decrease in heterozygosity in the TW_2 population, due to a decrease in frequency of the 120 bp allele. Heterozygosities for this particular locus remained almost constant in the TW_1 and TW_3 populations during this period (see Fig. 2).

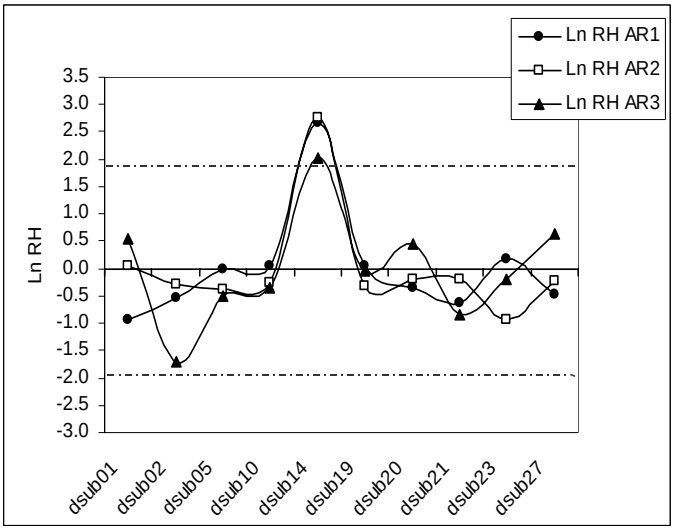

Figure 2A

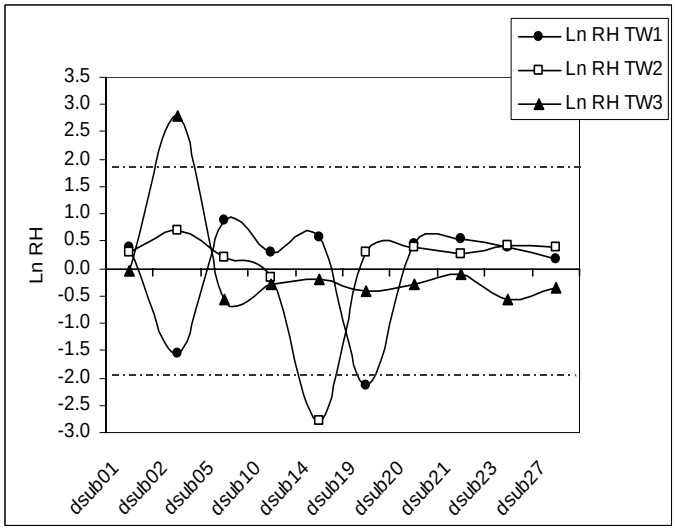

Figure 2B

Figure 2. Standardized Heterozygosity ratios (Ln RH) between generations 3 and 14 for AR (Fig. 2A) and TW (Fig. 2B) populations. Dashed lines represent the 95% confidence interval of the standardized normal distribution.

Comparisons of quantitative-character and molecular-genetic differentiation

Our previous studies showed clear laboratory adaptation in AR and TW populations over the first 40 generations of laboratory culture [8]. The temporal dynamics of phenotypic adaptation involved large, consistent directional changes in several life-history traits between generations 3 and 40, particularly fecundity-related traits (see Table 5). Thus we expected to observe $Q_{st}>F_{st}$ for these characters.

Quantitative-character and molecular-genetic differentiation contrasts (Q_{st} *vs.* F_{st}) between generations 3 and 40 were calculated for each replicate population (see Fig. 3). Neutral molecular-genetic differentiation was estimated excluding locus *dsub14* from the dataset. In the AR populations, early fecundity, peak fecundity, and female starvation resistance showed significantly higher differentiation than that obtained with molecular markers (one tailed *t*-test; $p<0.05$). In the TW populations, Q_{st} values for age of first reproduction, early fecundity, peak fecundity and male starvation resistance were all significantly higher than F_{st} (one tailed *t*-test; $p<0.05$).

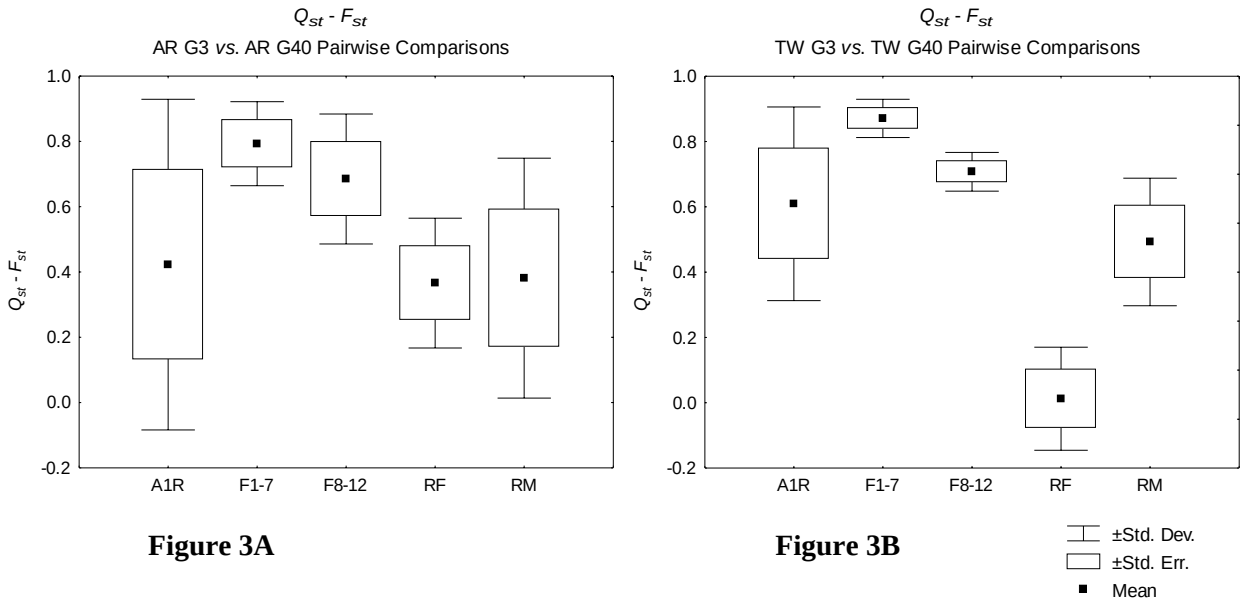

Figure 3A **Figure 3B**

Figure 3. Q_{st} minus F_{st} pairwise comparisons across generations 3 and 40. Comparisons for AR (Fig. 3A) and TW (Fig. 3B) populations. Q_{st} values were obtained for the following life-history traits: age of first reproduction (A1R), early fecundity (F1-7), peak fecundity (F8-12), female and male starvation resistance (RF and RM).

Table 5. Data of several life history traits for each AR and TW laboratory populations.

	A1R		F1-7		F8-12		RF		RM	
	G3	G40	G3	G40	G3	G40	G3	G40	G3	G40
AR_1	2.081	1.372	0.121	0.639	0.528	0.975	0.986	1.131	0.964	1.033
AR_2	2.161	0.893	0.082	0.894	0.432	1.029	0.818	0.991	0.772	1.051
AR_3	1.605	1.505	0.205	0.565	0.610	0.858	0.985	0.901	0.855	1.024
TW_1	1.620	1.250	0.301	0.819	0.638	1.062	0.981	1.069	0.974	1.145
TW_2	1.959	0.794	0.089	0.941	0.532	1.030	0.894	0.978	0.824	1.232
TW_3	2.069	1.228	0.018	0.696	0.515	1.027	0.937	0.957	0.892	1.081

Note.- The values are presented as ratios relative to a control population. The following life-history traits are shown: age of first reproduction (A1R), early fecundity (F1-7), peak fecundity (F8-12), female and male starvation resistance (RF and RM, respectively).

Quantitative-character and neutral genetic differentiation was also studied by comparing Q_{gp} and F_{gp} between generation 3 and 40 in each group of populations (AR and TW) - see details in the Methods section. Quantitative differentiation Q_{gp} values for AR populations between generations 3 and 40 were not significantly different from neutral genetic differentiation (F_{gp}=0.220; CI=-0.068;0.508) for any of the life-history traits analyzed (see Table 6). For TW populations, only Q_{gp} for peak fecundity (F8-12) was significantly higher than neutral genetic differentiation (F_{gp}=0.257; CI=-0.046;0.561). Nevertheless, quantitative-character measures of differentiation were consistently higher, except for female starvation resistance for which we have not found consistent patterns of phenotypic adaptation [8]. On the other hand, as expected for neutral markers, F_{gp} estimates between generations were not significantly different from zero, for either AR or TW populations.

Table 6. Q_{gp} estimates between generations 3 and 40 for AR and TW populations.

Trait	Q_{gp}	
	AR	TW
A1R	0.577	0.760
Lower CI (95%)	-0.120	0.074
Upper CI (95%)	0.788	0.880
F1-7	0.866	0.874
Lower CI (95%)	0.323	0.377
Upper CI (95%)	0.933	0.937
F8-12	0.910	1.028
Lower CI (95%)	0.363	0.766
Upper CI (95%)	0.955	1.014
RF	-0.031	0.327
Lower CI (95%)	-0.219	-0.246
Upper CI (95%)	0.482	0.663
RM	0.630	0.832
Lower CI (95%)	-0.120	0.108
Upper CI (95%)	0.815	0.916

Note.- Q_{gp} estimates were obtained from phenotypic assays performed at generations 3 and 40 of both AR and TW populations.

Discussion

Initial microsatellite variability and genetic differentiation

Both AR and TW populations had similar high levels of initial variability at the ten microsatellite loci studied. Sampling effects of foundation thus apparently did not greatly deplete genetic variability at the start of laboratory culture. The mean allele number (n_A=13.4-14.9) and expected heterozygosity (H_{exp}=0.877-0.898) in these populations were similar to the values observed for the same seven common loci studied in other European natural populations (n_A=14-16.5, H_{exp}=0.875-0.911, data from [28]).

Nevertheless, AR and TW populations showed significant initial genetic differentiation. The overall F_{st} value of 0.016 at generation 3 was slightly higher than the values obtained from other comparisons among European populations of *Drosophila subobscura* (average F_{st} = 0.006; see [28]). This suggests independent evolution of the ancestral natural populations at the locations from which these populations were derived, Arrábida and Sintra, both in Portugal. These results are somewhat surprising, given the close proximity of the two natural sites where the founders were collected, with a distance of around 50 km. It is possible that the foundation process and the subsequent three generations in the laboratory environment may have accentuated the differences in allele frequencies between these populations. Further sampling and also the analyses of founder individuals may help to clarify whether these two natural populations present restricted gene flow or their genetic differentiation was an artefact of laboratory foundation.

Temporal dynamics of microsatellite variability and genetic differentiation

During the course of 40 generations of laboratory culture, the initially high genetic variability was progressively eroded: both allele number and heterozygosity showed signs of decline during this period. This was predictable since two forces that are both expected to erode genetic variability - random genetic drift and sustained directional selection – are likely to be pronounced in laboratory cultures, particularly given that effective population sizes are much lower than those occurring in natural populations of *Drosophila*. This depletion in genetic variability was generally observed throughout laboratory culture for both AR and TW populations, as shown by the parallel declines among variability indexes. However, it is important to note that this loss of genetic

variability was relatively mild, since after 40 generations of laboratory adaptation AR and TW populations retained, respectively, 95% and 90% of their initial genetic diversity. The careful maintenance and overall high census sizes (around 900 individuals) in our populations may explain these results. This is in accordance with the high levels of genetic variability that we had already found for our NW *Drosophila subobscura* populations after 49 generations in the laboratory, with 87 to 89% of the genetic diversity of the third generation of TW populations (Simões et al. unpublished data).

In a recent experimental evolution study with *Drosophila melanogaster* [10], a significant decline in heterozygosity was found, with an estimated loss of 16% of genetic variability in experimental populations maintained with a census size (N) of 100 individuals during 38 generations of laboratory culture after sampling from the wild. The relatively modest decline in genetic diversity observed in our populations (5-10%) also suggests the absence of important bottlenecks events in their laboratory evolution.

The two variability measures used in this study showed similar patterns of decline. Both average allele number and expected heterozygosity showed a non-linear pattern, with a higher rate of decline in genetic variability between generations 3 and 14. This decline might be the result of a high initial loss of rare alleles due to a bottleneck effect associated with the foundation from the wild. Such bottleneck effects are expected to lead to a large drop in allele number, though they are not expected to have a major impact on the rate of decline of average heterozygosity (see [29,30]). The slowing down of the rate of heterozygosity decline through time may instead be a result of a smaller effective population size during the initial generations of laboratory adaptation (see next section).

As a consequence of differential allele loss and allele frequency changes in each population, genetic differentiation (as measured by F_{st} values) increased among all six populations through time. This is expected to be particularly important in smaller populations, due to genetic drift [6]. In spite of the significant initial differentiation between AR and TW, there was a higher increase of genetic variance component between replicate populations within each group than between AR and TW groups through laboratory adaptation. This suggests that differences in the initial genetic background did not play an important role in the temporal divergence observed among our laboratory populations. This suggestion is reinforced by the very similar F_{st} values

obtained when comparing TW or AR populations within each group between generations 3 and 40 (average F_{st} values of 0.053 and 0.045, respectively), with the cross-groups comparison of F_{st} values between TW at generation 3 and AR at generation 40 and *vice-versa* (with an average F_{st} of 0.046 and 0.055, respectively). These results are similar to the values reported in a previous study (F_{st} of 0.064) comparing TW populations at their 3rd generation and NW populations at their 49th generation after foundation, the latter populations founded from the same natural location (Sintra) in 1998 (Simões et al. unpublished data).

Thus all our results indicate general similarity in the evolutionary dynamics of microsatellite loci during laboratory adaptation across foundations, whether these differ in space or time.

No consistent relationship was found between microsatellite variability levels and the adaptive dynamics of our laboratory populations. In fact, we found no association between the initial genetic variability in molecular markers – which were similar in both sets of populations - and the subsequent phenotypic evolutionary response to the laboratory environment – with a higher adaptive rate for TW relative to AR populations, particularly in the first 14 generations [8]. Furthermore, the depletion of genetic variability among molecular markers showed only a weak association with the phenotypic evolution of our populations. There was only a suggestion of a higher rate of depletion of heterozygosity in TW populations, which were the ones that presented a higher adaptive rate. Overall, the data suggest that phenotypic adaptation within our laboratory populations had little correlation with the variability shown by molecular markers. This suggests caution when applying microsatellite data to the analysis of adaptation, particularly in studies of the relationship between genetic variation and evolutionary potential (see also [31,32,33]; but see [34]).

Effective population sizes during laboratory adaptation

We have evidence of increase in the effective size of our laboratory populations through time. The higher selective pressures suffered shortly after laboratory foundation may account for the initial lower effective sizes, since family contributions may vary greatly under strong selection in an initial phase of adaptation [6]. It is however possible that a smaller N_e in the first period was in part due to the loss of rare alleles. Nevertheless, the pseudo-likelihood method of N_e estimation that we employed is considered less

sensitive to frequency changes in rare alleles [35]. Thus, it seems unlikely that the changes in effective population size found using this method were chiefly due to the effects of genetic drift in the first generations leading to the loss of rare alleles.

Average N_e values for the TW populations were systematically lower than those obtained for AR populations. In particular, TW populations presented a significantly lower effective population size when it was estimated using the loss of heterozygosity method applied to the first 14 generations. These results are consistent with the finding of both higher selective pressure (associated with a higher adaptive rate – see [8]) and more genetic drift in TW populations (see above).

The N_e/N ratios obtained in this study - 0.25 for AR and 0.21 for TW populations – are higher than most estimates based on laboratory-maintained populations. For instance, N_e/N values below 0.051 were found for captive populations of *Drosophila melanogaster* [36], and other studies in laboratory *Drosophila* populations have also presented values considerably below our estimate (see [36] for a brief review). Our N_e/N ratios were also higher than the average values of 0.11 reported for natural populations [37]. These higher values might be a result of the lower fluctuations in the overall census size of our laboratory populations through time, compared to other laboratory studies or what is expected to occur in wild populations.

Testing for positive selection at the molecular level

Both AR and TW populations are undergoing adaptation to laboratory conditions with respect to life-history traits, some of which show clear directional trends of improvement throughout laboratory adaptation [8]. For the microsatellite data, we obtained clear deviations from neutral expectations at locus *dsub14* for both AR and TW populations after 14 generations of laboratory adaptation, suggesting thus a selective sweep has occurred in the neighbouring region of this microsatellite. However, a significant increase in heterozygosity through time was observed at this locus, not a decline. This increase is possibly a transient effect due to the increase in frequency of an allele with low initial frequency at a locus with low overall variability (H_{exp}=0.286 for AR; H_{exp}=0.285 for TW, at generation 3; [see also Additional files 1, 2 and 3]). The lower initial variability in this locus could itself have been the result of selective constraints affecting this region in wild populations, although we cannot exclude low

mutation rates as a possible explanation given the low number of repeats in this locus [38].

A strong point in favour of the involvement of directional selection near the *dsub14* locus is that the allele showing an increased frequency was the same in all TW populations. However, this pattern is not ineluctable, since sampling effects in the formation of our replicate populations, particularly involving low frequency alleles, could have led to different linkage disequilibria of alleles at this microsatellite locus with positively selected alleles some distance away from *dsub14*. This may explain the pattern observed in the AR populations, where two alleles were involved, one common to the AR_1 and AR_2 populations, and a different one for AR_3.

Between generations 14 and 40, microsatellite locus *dsub14* showed a significant deviation from neutrality in AR but not in TW populations. During this period, the TW_2 population actually undergoes a drop in the frequency of the putative hitchhiking allele, leading to a significant decline in heterozygosity over this period. Moreover, the deviation from neutrality of the AR_3 population was not due to changes of frequency in the expected direction, since there was a drop in frequency of the putatively selected allele. These results are not the predicted outcome, since hitchhiking within a region undergoing directional selection is expected to lead to a consistent increase in the frequency of the hitchhiking allele and ultimately to its fixation, unless linkage is broken by recombination.

The continued monitoring of allele frequency change at this locus over subsequent generations could help to clarify the evolutionary forces acting on it. Also, the analysis of other microsatellite loci adjacent to this particular locus, searching for signs of low polymorphism in the genomic region, may reveal the extent of selective pressures in that region. At the same time, sequence analysis of flanking regions could be useful in the search for candidate genes underlying phenotypic adaptation. In fact, its location in chromosome O could account for the hitchhiking effect involving *dsub14,* since this chromosome harbours considerable inversion polymorphisms in *Drosophila subobscura*, which limit recombination [39,40].

The indication of a selective sweep from a survey of just a relatively modest number of loci suggests that the impact of directional selection on the genome, even at non-coding regions, is not negligible. This is in agreement with recent published studies

(e.g., [41]) showing that a large portion of the non-coding DNA of *Drosophila melanogaster* might be suffering the effects of adaptive evolution.

Adaptive *versus* neutral divergence

Under directional selection, the rate of differentiation is expected to be higher than that occurring due to merely neutral causes [11,12,42]. The evolutionary changes between generation 3 and 40 for both TW and AR populations are in accordance with this expectation, with quantitative character differentiation (Q_{st}) being higher than neutral genetic differentiation (F_{st}), particularly for fecundity traits. Other empirical studies have also tested successfully the assumption of $Q_{st}>F_{st}$ as an indication of the relevance of selective forces in promoting population divergence [20,43]. This $Q_{st}>F_{st}$ result has also been found in several empirical studies of natural populations (e.g., [16,17,18,44]).

However, when applying a more conservative approach, using Q_{gp} and F_{gp} measures of differentiation among generations at the group level, only one significantly higher Q_{gp} value was obtained in our study, that for peak fecundity in TW populations. The broadness of the parametric Confidence Intervals (CIs) found here partially explains these results. This problem has also been addressed in another study comparing quantitative and neutral genetic differentiation measures in laboratory populations [20], pointing out that the low precision of parametric CIs may prevent correct evolutionary inferences. Our data support that conclusion too. Using this approach, no consistent evidence of directional natural selection was found in our populations, a conclusion that is incorrect given our knowledge of the evolutionary history of the laboratory populations involved. This suggests that low statistical power and lack of precision can be an important caveat for this approach (see [45]). In this sense, absence of significant differences between quantitative and molecular measures should be treated cautiously and not immediately interpreted as reflecting an absence of selective forces acting on natural populations, or even similarity between selection and drift effects.

Conclusions

We observed a depletion of genetic variability and an increase in genetic differentiation among our laboratory populations through time. This is the predicted outcome of genetic drift effects in populations with smaller sizes, relative to those that are

characteristic in the natural environment. Different genetic backgrounds appear to have had limited impact on these drift effects, since laboratory populations founded from different wild sources did not differ in their rate of variability decline through time. We found evidence that selection acting on life history traits interacts with genetic drift, particularly through the smaller effective population sizes at early stages of adaptation, leading to a steeper initial drop in molecular genetic variability.

We also found evidence of positive selection at one of the ten molecular markers analyzed. Given our meager sampling, it is reasonable to assume that adaptation can affect a relevant portion of the genome even at neutral sites due to genetic hitchhiking.

Our comparison of Q_{st} with F_{st} estimates indicates that this is a valid approach to produce correct inferences of directional selection though it also illustrates the need to improve the statistical approaches involved.

Finally, we found no clear association between overall measures of microsatellite genetic diversity and life-history adaptation. This suggests that inferences of evolutionary potential for adaptation based on microsatellite variability should be made with caution. However, this study also shows that selective processes might influence patterns of molecular genetic variability and differentiation. The combined analysis of molecular genetic and life-history data can be a powerful approach to help us understand the evolutionary mechanisms of local adaptation.

Methods

Foundation and maintenance of the laboratory populations

This study involves two synchronous laboratory foundations carried out in autumn 2001, one from Sintra, Portugal, called "TW", and another from Arrábida, Portugal, called "AR" (the two localities being 50 Km apart). The TW population was founded from 110 females and 44 males and the AR population began with 59 females and 24 males. After two generations in the laboratory, the immediate descendants of these samples were each split into three replicate populations, $TW_{1\text{-}3}$ and $AR_{1\text{-}3}$. From the moment the populations were brought into the laboratory, they were all maintained under the same conditions: discrete generations of 28 days, reproduction close to peak fecundity, a controlled temperature of 18ºC, and controlled densities (see [8,46]). Population sizes were usually between 600 and 1200 individuals.

Microsatellite genotyping methods

AR and TW populations were genotyped for 10 microsatellite loci at generations 3, 14, and 40 from foundation. At each generation, 30 females were analyzed for each of the six populations studied (TW_{1-3} and AR_{1-3}).

The ten microsatellite loci analyzed in this study were: *dsub01*, *dsub02*, *dsub05*, *dsub10*, *dsub14*, *dsub19*, *dsub20*, *dsub21*, *dsub23* and *dsub27*. These markers were previously identified and characterized [38]. Loci *dsub05*, *dsub19* and *dsub21* are X-linked and the others are autosomal.

DNA for the microsatellite analysis was extracted from single flies using an extraction protocol described in [47]. PCR reactions were performed for a total volume of 25 µl with 2.5 pmol of each primer (10µM), 3 µl dNTP´s (1mM), 2 µl 10 x buffer, 1 U *Taq* polymerase and 1 µl of DNA. All 10 loci were amplified using four different multiplex PCR reactions *(dsub02+dsub05; dsub10+dsub14; dsub20+dsub21+dsub27; dsub01+dsub19+dsub23).* All reactions were performed on an ABI GeneAmp PCR System 2700 machine using the following steps: 5 min at 95ºC, then 30 cycles of 1 min at 95ºC, 1 min at 54ºC and 30 s at 72ºC followed by 5 min at 72ºC. After amplification, the products were visualized in an agarose gel and then loaded on an ABI PRISM 310 sequencer (Applied Biosystems). Allele sizes were estimated by comparison to an internal size standard (GeneScan-500 ROX) using the software program Genotyper (Applied Biosystems).

Life-History traits

The life history traits used in this study were the following: age of first reproduction (A1R), early fecundity (fecundity between days 1 and 7 of adult life – F1-7), peak fecundity (fecundity between days 8 and 12 of adult life – F8-12) and male and female starvation resistance – RM and RF, respectively (for assay details see [46,48]). Data for these life-history traits were obtained from phenotypic assays performed at generations 3 and 40 of AR and TW laboratory adaptation (see Table 5). Assays were performed using mated pairs, with samples sizes ranging from 14 to 25 pairs per replicate population. AR and TW phenotypic assays included simultaneous controls using long-established NB *D. suboscura* laboratory populations as reference populations [8].

Statistical methods

Microsatellite Analysis

a) Measures of genetic diversity and differentiation

Genetic variability was measured using both mean number of alleles per locus and mean expected heterozygosity with GENEPOP, version 3.2 [49].

Differences in genetic variability between AR and TW sets of populations in each generation were assessed using a bifactorial mixed ANOVA defining *group* (with two categories: AR and TW) as a fixed factor and *locus* as a random factor, with each genetic variability measure as a dependent variable. To test for differences in genetic variability in each group across generations, we applied a similar model, with *generation* as a fixed factor (with three categories: generations 3, 14, and 40) and *locus* as a random factor. The changes in microsatellite variability through time were studied by defining two *periods*: the first period between generations 3 and 14 and the subsequent period between generations 14 and 40. Rates of variability decline were calculated for each period for both AR and TW populations, using both allele number and expected heterozygosity (standardized by the square root of the number of generations of each period). Differences in the rates of variability decline between *periods* and *groups* were tested with trifactorial mixed ANOVAs (sigma-restricted, type III SS model) with *group*, *period* (fixed) and *locus* (random) as factors.

All parameters tested by ANOVA had a normal distribution of residuals. Rates of heterozygosity decline were arcsine transformed to meet ANOVA assumptions. All ANOVAs were performed using Statistica 5.0.

To estimate genetic differentiation, F_{st} values were calculated using ARLEQUIN, version 2.000 [50]. Pairwise F_{st} values and corresponding significance values (calculated by permutation) were obtained for each set of populations (AR or TW) by comparing replicate populations within and across generations. To account for multiple testing, we used the sequential Bonferroni method [51]. Within each particular set of populations, the significance of the differences between F_{st} values across generations was assessed by 10000 permutations using the FSTAT program [52]. Differentiation across generations between sets of populations (AR or TW) was evaluated by *t*-tests using as data points the 3 AR *vs.* TW pairwise F_{st} values between same-numbered replicate populations, after testing for normal distribution of residuals.

Hierarchical analyses of molecular variance (AMOVA) were performed to analyze the partition of total genetic variance into covariance components due to three different sources of variation: among groups (or generations), among populations/within groups (or generations) and within populations. Several AMOVA analyses were performed for the following comparisons: (a) Among groups: AR populations *vs.* TW populations in each of the three generations analyzed (generations 3, 14 and 40); (b) Among generations: TW populations at generation 3 *vs.* TW populations at generation 40; or AR populations at generation 3 *vs.* AR populations at generation 40. ARLEQUIN version 2.000 was used for all these analyses.

b) Estimating effective population sizes

Effective population sizes (N_e) for each AR and TW population during laboratory adaptation were estimated from temporal microsatellite data using a pseudo-likelihood approach (Wang 2001) and also through the loss of heterozygosity formula $H_t/H_0=(1-1/2N_e)^t$ (see [53]). Likelihood-based methods were used because they provide more reliable N_e estimates relative to classical methods (e.g., [54,55]), particularly for samples with many rare alleles [56,57].

Effective population sizes were estimated for the two periods previously referred to (between generations 3 -14 and between generations 14-40), and also for the overall data (generations 3 to 40) in both AR and TW populations.

The pseudo-likelihood N_e estimates were obtained using the MLNE program [35,58], given our temporally spaced samples for each AR and TW population. All analyses were performed allowing a maximum N_e value of 1000.

c) Testing for positive selection

Effects of positive selection were tested for each microsatellite locus by applying the Ln RH test statistic [22]. This test is based on the comparison of the logarithm of the ratio between expected heterozygosities obtained for each locus in two populations: $\text{Ln RH} = \text{Ln}\ [((1/(1- H_{pop1}))^2 -1)/((1/(1- H_{pop2}))^2 -1)]$. The aim of this test is to search for loci with a pattern of variability which is significantly different from that expected with neutrality.

To apply this test, ratios of expected heterozygosities were calculated for each locus using data from generations 3 and 14 (G14/G3 ratios) and also generations 14 and

40 (G40/G14 ratios) for each AR and TW populations. To account for the different effective population sizes of X chromosomes, a correction was introduced for the X chromosomal loci heterozygosities (see [59]):

$$H_{corr}=1-1/[\sqrt{1+k(1/(1-H_{obs})^2-1)}] ,$$

the correction factor k used was 1.33, assuming a balanced sex ratio [60]. Since Ln RH values are expected to follow a Z distribution for neutrally evolving microsatellite loci [21], significant deviations of standardized Ln RH values from this distribution indicate a putative selective sweep [22]. This test was applied for each AR and TW replicate population.

To detect potentially selected loci, we also performed a one-way ANOVA, defining *locus* as factor and the Ln RH values (of the three AR or TW populations) as the dependent variable. To search for differences between loci a *post hoc* Scheffé test was also performed. Normality in Ln RH data was previously tested. All these analyses were done in Statistica 5.0.

Estimating quantitative versus molecular genetic divergence

The degree of temporal quantitative differentiation for life history traits between generations 3 and 40 after laboratory foundation was analyzed at two levels: (a) For each replicate population, Q_{st} values were calculated from the variance between generations and the variance within generations (among individuals); (b) For each group of replicate populations (AR or TW), Q_{gp} values involved the estimation of the variance between generations and the variance within generations among replicate populations. Estimates of quantitative differentiation were obtained for five life-history traits: age of first reproduction (A1R), early and peak fecundity (F1-7 and F8-12, respectively), as well as male and female starvation resistance (RM and RF).

Specifically, Q_{st} values - obtained for each of the AR and TW populations - were calculated as $V_b/(V_b+2V_w)$ following [14], with V_b being the variance distributed among generations and V_w the variance component within generations. The phenotypic variation among generations was studied using a one-way ANOVA performed for each population (e.g. AR_1 G3 *vs.* AR_1 G40). For each replicate population, the between-generation variance component was obtained by equating observed mean squares (MS) with the expected values. The within-generation variance component was obtained directly from the observed mean square of the error.

To minimize the environmental component of phenotypic variation in V_w, this component was multiplied by 0.2, a common estimate of the heritability for life-history traits among laboratory populations of *Drosophila* [61,62]. However, the incomplete extraction of environmental effects on the V_w component would lead to lower Q_{st} estimates, making this a conservative test of the importance of directional natural selection in promoting population differentiation when using the criterion $Q_{st}>F_{st}$. Since it is impossible to perform synchronous phenotypic assays across generations, the raw data obtained in each assay were divided by the mean value of the simultaneously-assayed control (NB) population having the same arbitrarily assigned number (e.g. AR_1/NB_1), prior to calculating Q_{st} estimates. Statistical comparisons of Q_{st} and F_{st} pairwise values for each population were performed by means of a *t*-test using as data the differences between Q_{st} and F_{st} values obtained for each replicate population (e.g. AR_1 G3 *vs.* AR_1 G40) after confirming the normal distribution of residuals.

Quantitative-character differentiation and molecular-genetic differentiation between generations 3 and 40 were also studied by comparing Q_{gp} and $F_{gp.}$ The variance components – V_a (variance among generations), V_b (variance among the three replicates within generations) and V_w (variance among individuals within replicate populations) – were estimated using a two-way ANOVA, with the replicate population factor nested within generations. The degree of quantitative-character differentiation - Q_{gp} – was calculated for each of the five life-history traits assayed as $V_a/(V_a+2V_b)$. This measure, unlike Q_{st}, includes the heterogeneity among replicate populations within generations in the denominator, thus minimizing the effects of stochastic evolutionary divergence due to genetic drift alone. This is similar to the approach used in [20], though in their case the V_w (within-population variance) component was included in the denominator. Our approach minimized the problem of using phenotypic variance to estimate variance quantitative-character differentiation, since the V_a and V_b components are not expected to include a relevant environmental component, at least in comparison to the V_w component. Confidence intervals (95%) for Q_{gp} were calculated using an equation in [63, p.563], adjusted to our particular case. Specifically, the coefficient 4 (applied to heritability estimates) was replaced by $K=(V_a+V_b)/(V_a+2*V_b)$, in each of our CI estimates:

$$K*((F/F_U)-1)/((F/F_U)+n-1) < Q_{gp} < K*((F/F_L)-1)/((F/F_L)+n-1)$$

with n=3 (the number of populations within each group), *F* being the observed *F* statistics, and F_L and F_U the lower and upper *F* values. These values were calculated as: $F_U=F_{1,\ 4,\ 0.025}$; and $F_L=1/F_{4,\ 1,\ 0.025}$ (with N-1=1 and T-N=4 degrees of freedom: in our case N=2, our two groups, and T=6, the total number of populations).

The parameter F_{gp} was estimated as $V_a/(V_a + V_b)$, with V_a being the variance component among generations and V_b the variance among replicate populations within generations, as obtained by a locus-by-locus AMOVA applied to the molecular data. Given that the V_b component is expect to contain most or all of the variance due to genetic drift, F_{gp} is likely to approach zero in neutral markers. Approximate confidence intervals for the average F_{gp} were obtained from the standard error of the estimates of F_{gp} for each locus after testing for normality.

Authors' contributions

PS, JS and MM performed the life history trait assays and maintained laboratory populations. PS and JS performed the microsatellite analyses. PS and MM carried out the statistical analyses. PS, MP, MRR and MM designed the experiment. PS and MM wrote the first draft of the manuscript. MP, MRR, and JS contributed to the final draft of the manuscript. All authors read and approved the final manuscript.

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Additional files

Additional file 1 - Genetic Variability of AR and TW populations at generation 3.
Average allele number and expected heterozygosity per locus for the AR and TW sets of populations at generation 3 of laboratory evolution.

Additional file 2 - Genetic Variability of AR and TW populations at generation 14.
Average allele number and expected heterozygosity per locus for the AR and TW sets of populations at generation 14 of laboratory evolution.

Additional file 3 - Genetic Variability of AR and TW populations at generation 40.
Average allele number and expected heterozygosity per locus for the AR and TW sets of populations at generation 40 of laboratory evolution.

Additional file 4 - Pairwise F_{st} comparisons between AR and TW populations.
Genetic differentiation between AR and TW populations at each of the three generations analyzed: generation 3, 14 and 40.

Additional file 5 - Pairwise F_{st} comparisons within and across laboratory generations.
Genetic differentiation in each set of replicate populations (AR or TW) within and across generations analyzed.

Table A1. Genetic Variability of AR and TW populations at generation 3.

Regime	Population	Locus	Chromosome	N_a	H_{EXP}
AR	1	dsub01	O	9	0.793
AR	1	dsub02	O	12	0.836
AR	1	dsub05	A	20	0.946
AR	1	dsub10	U	16	0.908
AR	1	dsub14	O	4	0.252
AR	1	dsub19	A	11	0.868
AR	1	dsub20	E	18	0.921
AR	1	dsub21	A	11	0.897
AR	1	dsub23	J	13	0.861
AR	1	dsub27	J	12	0.875
AR	2	dsub01	O	9	0.828
AR	2	dsub02	O	14	0.862
AR	2	dsub05	A	18	0.936
AR	2	dsub10	U	18	0.944
AR	2	dsub14	O	3	0.330
AR	2	dsub19	A	12	0.859
AR	2	dsub20	E	24	0.956
AR	2	dsub21	A	12	0.890
AR	2	dsub23	J	11	0.855
AR	2	dsub27	J	11	0.854
AR	3	dsub01	O	10	0.859
AR	3	dsub02	O	13	0.867
AR	3	dsub05	A	22	0.950
AR	3	dsub10	U	17	0.914
AR	3	dsub14	O	5	0.276
AR	3	dsub19	A	12	0.886
AR	3	dsub20	E	21	0.949
AR	3	dsub21	A	11	0.888
AR	3	dsub23	J	14	0.832
AR	3	dsub27	J	11	0.866
TW	1	dsub01	O	11	0.898
TW	1	dsub02	O	16	0.888
TW	1	dsub05	A	21	0.942
TW	1	dsub10	U	16	0.916
TW	1	dsub14	O	4	0.307
TW	1	dsub19	A	12	0.851
TW	1	dsub20	E	19	0.946
TW	1	dsub21	A	13	0.894
TW	1	dsub23	J	13	0.844
TW	1	dsub27	J	11	0.860
TW	2	dsub01	O	10	0.881
TW	2	dsub02	O	17	0.912
TW	2	dsub05	A	20	0.920
TW	2	dsub10	U	20	0.940
TW	2	dsub14	O	3	0.327
TW	2	dsub19	A	12	0.866
TW	2	dsub20	E	22	0.949
TW	2	dsub21	A	10	0.876
TW	2	dsub23	J	13	0.830
TW	2	dsub27	J	13	0.882
TW	3	dsub01	O	10	0.901
TW	3	dsub02	O	16	0.910
TW	3	dsub05	A	19	0.915
TW	3	dsub10	U	20	0.946
TW	3	dsub14	O	3	0.220
TW	3	dsub19	A	12	0.860
TW	3	dsub20	E	24	0.953
TW	3	dsub21	A	10	0.865
TW	3	dsub23	J	14	0.838
TW	3	dsub27	J	12	0.872

Note.- N_a - Allele number; H_{EXP} - Expected Heterozygosity

Table A2. Genetic Variability of AR and TW populations at generation 14.

Regime	Population	Locus	Chromosome	N_a	H_{EXP}
AR	1	dsub01	O	8	0.806
AR	1	dsub02	O	8	0.763
AR	1	dsub05	A	13	0.910
AR	1	dsub10	U	13	0.896
AR	1	dsub14	O	5	0.504
AR	1	dsub19	A	10	0.815
AR	1	dsub20	E	14	0.880
AR	1	dsub21	A	9	0.822
AR	1	dsub23	J	9	0.767
AR	1	dsub27	J	10	0.880
AR	2	dsub01	O	8	0.738
AR	2	dsub02	O	10	0.828
AR	2	dsub05	A	11	0.872
AR	2	dsub10	U	14	0.918
AR	2	dsub14	O	3	0.464
AR	2	dsub19	A	10	0.885
AR	2	dsub20	E	17	0.930
AR	2	dsub21	A	8	0.814
AR	2	dsub23	J	11	0.789
AR	2	dsub27	J	10	0.832
AR	3	dsub01	O	8	0.805
AR	3	dsub02	O	10	0.866
AR	3	dsub05	A	15	0.923
AR	3	dsub10	U	17	0.919
AR	3	dsub14	O	3	0.405
AR	3	dsub19	A	10	0.853
AR	3	dsub20	E	16	0.883
AR	3	dsub21	A	10	0.865
AR	3	dsub23	J	13	0.791
AR	3	dsub27	J	8	0.812
TW	1	dsub01	O	9	0.865
TW	1	dsub02	O	10	0.758
TW	1	dsub05	A	11	0.793
TW	1	dsub10	U	12	0.874
TW	1	dsub14	O	2	0.494
TW	1	dsub19	A	7	0.720
TW	1	dsub20	E	18	0.921
TW	1	dsub21	A	11	0.796
TW	1	dsub23	J	12	0.897
TW	1	dsub27	J	9	0.796
TW	2	dsub01	O	11	0.888
TW	2	dsub02	O	13	0.777
TW	2	dsub05	A	11	0.876
TW	2	dsub10	U	11	0.752
TW	2	dsub14	O	4	0.535
TW	2	dsub19	A	8	0.834
TW	2	dsub20	E	14	0.893
TW	2	dsub21	A	10	0.872
TW	2	dsub23	J	12	0.857
TW	2	dsub27	J	12	0.837
TW	3	dsub01	O	9	0.771
TW	3	dsub02	O	9	0.447
TW	3	dsub05	A	10	0.868
TW	3	dsub10	U	12	0.873
TW	3	dsub14	O	2	0.413
TW	3	dsub19	A	10	0.835
TW	3	dsub20	E	19	0.930
TW	3	dsub21	A	9	0.773
TW	3	dsub23	J	12	0.831
TW	3	dsub27	J	11	0.863

Note.- N_a - Allele number; H_{EXP} - Expected Heterozygosity

Table A3. Genetic Variability of AR and TW populations at generation 40.

Regime	Population	Locus	Chromosome	N_a	H_{EXP}
AR	1	dsub01	O	7	0.725
AR	1	dsub02	O	8	0.719
AR	1	dsub05	A	14	0.890
AR	1	dsub10	U	11	0.889
AR	1	dsub14	O	4	0.593
AR	1	dsub19	A	7	0.811
AR	1	dsub20	E	12	0.823
AR	1	dsub21	A	7	0.757
AR	1	dsub23	J	11	0.773
AR	1	dsub27	J	8	0.812
AR	2	dsub01	O	6	0.743
AR	2	dsub02	O	9	0.781
AR	2	dsub05	A	8	0.803
AR	2	dsub10	U	12	0.846
AR	2	dsub14	O	4	0.598
AR	2	dsub19	A	7	0.821
AR	2	dsub20	E	14	0.867
AR	2	dsub21	A	8	0.785
AR	2	dsub23	J	5	0.687
AR	2	dsub27	J	8	0.795
AR	3	dsub01	O	7	0.812
AR	3	dsub02	O	7	0.791
AR	3	dsub05	A	12	0.868
AR	3	dsub10	U	15	0.873
AR	3	dsub14	O	4	0.418
AR	3	dsub19	A	8	0.836
AR	3	dsub20	E	13	0.890
AR	3	dsub21	A	8	0.815
AR	3	dsub23	J	8	0.775
AR	3	dsub27	J	7	0.823
TW	1	dsub01	O	7	0.832
TW	1	dsub02	O	8	0.538
TW	1	dsub05	A	15	0.903
TW	1	dsub10	U	12	0.818
TW	1	dsub14	O	2	0.499
TW	1	dsub19	A	6	0.483
TW	1	dsub20	E	13	0.901
TW	1	dsub21	A	9	0.807
TW	1	dsub23	J	11	0.864
TW	1	dsub27	J	7	0.738
TW	2	dsub01	O	10	0.822
TW	2	dsub02	O	12	0.844
TW	2	dsub05	A	13	0.794
TW	2	dsub10	U	9	0.645
TW	2	dsub14	O	2	0.325
TW	2	dsub19	A	6	0.788
TW	2	dsub20	E	14	0.862
TW	2	dsub21	A	8	0.802
TW	2	dsub23	J	10	0.838
TW	2	dsub27	J	11	0.814
TW	3	dsub01	O	8	0.812
TW	3	dsub02	O	7	0.724
TW	3	dsub05	A	8	0.780
TW	3	dsub10	U	10	0.847
TW	3	dsub14	O	3	0.414
TW	3	dsub19	A	7	0.790
TW	3	dsub20	E	14	0.900
TW	3	dsub21	A	7	0.794
TW	3	dsub23	J	9	0.756
TW	3	dsub27	J	9	0.826

Note.- N_a - Allele number; H_{EXP} - Expected Heterozygosity

Table A4. Pairwise F_{st} comparisons between AR and TW populations.

	AR_1 G3	**AR_2 G3**	**AR_3 G3**
TW_1 G3	0.017	0.012	0.011
TW_2 G3	0.021	0.013	0.014
TW_3 G3	0.020	0.015	0.016
	AR_1 G14	**AR_2 G14**	**AR_3 G14**
TW_1 G14	0.091	0.087	0.069
TW_2 G14	0.073	0.065	0.055
TW_3 G14	0.065	0.079	0.057
	AR_1 G40	**AR_2 G40**	**AR_3 G40**
TW_1 G40	0.110	0.140	0.135
TW_2 G40	0.105	0.120	0.098
TW_3 G40	0.061	0.106	0.081

Note.- All pairwise comparisons are highly significant (p<0.01).

Table A5. Pairwise F_{st} comparisons within and across laboratory generations.

	AR_1 G3	AR_2 G3	AR_3 G3	AR_1 G14	AR_2 G14	AR_3 G14	AR_1 G40	AR_2 G40	AR_3 G40
AR_1 G3	-	0.008	0.003	0.022	0.019	0.013	0.043	0.058	0.024
AR_2 G3	n.s.	-	0.001	0.031	0.026	0.010	0.046	0.069	0.034
AR_3 G3	n.s.	n.s.	-	0.028	0.023	0.007	0.044	0.062	0.028
AR_1 G14	**	**	**	-	0.028	0.030	0.016	0.053	0.042
AR_2 G14	**	**	**	**	-	0.022	0.043	0.031	0.037
AR_3 G14	*	n.s.	n.s.	**	**	-	0.036	0.071	0.015
AR_1 G40	**	**	**	**	**	**	-	0.064	0.057
AR_2 G40	**	**	**	**	**	**	**	-	0.073
AR_3 G40	**	**	**	**	**	**	**	**	-

	TW_1 G3	TW_2 G3	TW_3 G3	TW_1 G14	TW_2 G14	TW_3 G14	TW_1 G40	TW_2 G40	TW_3 G40
TW_1 G3	-	0.003	0.000	0.032	0.024	0.043	0.061	0.040	0.050
TW_2 G3	n.s.	-	0.001	0.043	0.027	0.042	0.068	0.045	0.048
TW_3 G3	n.s.	n.s.	-	0.047	0.034	0.040	0.072	0.049	0.044
TW_1 G14	**	**	**	-	0.030	0.052	0.027	0.064	0.081
TW_2 G14	**	**	**	**	-	0.032	0.047	0.032	0.054
TW_3 G14	**	**	**	**	**	-	0.067	0.075	0.020
TW_1 G40	**	**	**	**	**	**	-	0.057	0.096
TW_2 G40	**	**	**	**	**	**	**	-	0.079
TW_3 G40	**	**	**	**	**	**	**	**	-

Note.- Below diagonal - Pairwise F_{st} values; Above diagonal - p values (n.s. - $p>0.05$; * - $0.01<p<0.05$; ** - $p<0.01$).

Chapter 7.
DISCUSSION

I will now summarize the major findings of this series of studies and also briefly discuss their contribution to a broader understanding of an ongoing adaptive process. I will specifically try to address the main questions raised in the Introduction, including short versus long-term adaptive patterns, repeatability of evolution in populations subjected to a uniform environment, as well as the molecular genetic changes that occur during laboratory adaptation. I will specifically highlight the value of molecular genetic techniques for the study of both evolutionary processes in general and evolution during captivity in particular. Finally, I will discuss the significance of this set of studies for management of populations in long-term *ex situ* conservation programmes.

I will end this thesis by pointing out some research approaches that might provide further insight in the study of adaptation and evolutionary processes in general.

Initial Adaptation

This study revealed diverse patterns of evolutionary response to laboratory environment, depending on the life-history traits analysed. A general pattern of improvement for fecundity traits was observed regardless of sampling or genetic background differences between populations (see Simões *et al.,* 2007a,b,c / chapters 2, 3 and 4). This is a somewhat expected outcome, since adaptation to a novel environment is expected to lead to functional improvements in traits tightly connected to fitness. Improvement in fitness related traits has been found in most studies of laboratory evolution studying adaptation to a novel environment in *Drosophila* (Matos *et al.,* 2000; 2002; Sgrò & Partridge, 2000; Gilligan & Frankham, 2003). This further suggests that natural populations of *Drosophila* tend to maintain genetic variability for fitness characters that can respond to selection when subject to a novel environment.

The evolution of improved performance during laboratory culture might also be expected for starvation resistance as a result of positive genetic covariance between fitness traits, at least during the initial generations in a novel environment (see Service & Rose, 1985). A suggestion of a biphasic pattern for female starvation resistance had been found in an earlier study performed by our team using the initial NW data (Matos

et al., 2002; Matos *et al.,* 2004). This was interpreted as suggesting temporal changes of genetic correlations from positive to negative values. However, female starvation resistance did not show any clear evolutionary pattern during laboratory culture in the populations studied here. For the populations introduced in 2001, a significant increase in female starvation resistance was observed for the TW populations during the first 40 generations of laboratory culture relative to the NB data. On the other hand, AR populations showed no improvement for this trait (see chapters 2 and 3). Furthermore, an analysis including data from the three sets of populations mentioned above (NW, TW and AR) and the four sets of populations founded in 2005 showed an overall negative trend for the evolutionary trajectories of this trait after the first 15-21 generations in the laboratory (see chapter 4). These results do not support a clear role for starvation resistance in the evolutionary processes leading to adaptation to the new laboratory environment. This can be explained by the absence of severe stressful conditions in the laboratory maintenance regime.

Starvation resistance has also shown disparate evolutionary patterns in other studies of laboratory adaptation (e.g. Sgrò & Partridge, 2000 *vs.* Griffiths *et al.,* 2005; see more examples in chapters 2 and 3). It is possible that these discrepancies between studies arise from differences in the initial genetic composition of the populations studied, as a result of their prior evolutionary history in the wild environment (Harshman & Hoffmann, 2000). Genetic variation between populations has been shown for starvation characters in *D. melanogaster*, although ecological studies addressing the impact of different stresses in field conditions are lacking (Hoffmann & Harshman, 1999). In this context, Hoffmann *et al.* (2001a) have found only weak geographical differentiation despite high intra-population variation, for this particular stress character in *D. melanogaster* populations.

The occurrence of discrepancies in laboratory studies relative to the starvation resistance evolutionary patterns is also more likely considering that natural selection in benign laboratory conditions might not be sufficient to override effects associated with prior genetic composition and evolutionary history (see chapter 4). Furthermore, the use of different methodological approaches to the study of laboratory adaptation (e.g. comparative method *vs*. evolutionary trajectories, see discussion below) may also contribute to differences between studies (Matos *et al.,* 2004).

Evolutionary changes in juvenile traits are also expected to occur during adaptation to a novel environment, given their direct association with life-cycle patterns.

In fact, juvenile traits, such as development time, have shown associations with adult traits, such as fecundity and lipid content (Chippindale *et al.*, 2004). However, the analysis of the evolutionary trajectories of juvenile characters, specifically juvenile viability and development time (from egg to eclosion), did not show any pattern of improvement during laboratory adaptation (see chapter 2). It is possible that juvenile development time during initial laboratory adaptation remains fairly stable as a result of an evolutionary compromise between the need for faster development - associated with a selective pressure for early maturation in the laboratory regime - and the potential drawbacks of early development, such as smaller size, and overall reduced fitness (Prasad & Joshi, 2003).

Long-term Laboratory Evolution

This study provides evidence for a deceleration in the evolutionary response over time. This was observed in the NW laboratory populations, which showed a slowing down of the evolutionary response after around 90 generations of laboratory adaptation, particularly for fecundity characters (see chapters 2 and 3). This was interpreted as populations reaching an evolutionary optimum for these specific traits, which will eventually lead to a complete cessation of improvement. In fact, this is what happened in our long established NB populations, which show no net evolutionary trend for fecundity traits after 176 generations of laboratory culture (see chapter 2 and 3). Ultimately, this deceleration of evolutionary response is expected as a result of depletion of additive genetic variability of fitness related traits, in accordance with Fisher´s Fundamental Theorem of Natural Selection (1930). Near the optimum the evolutionary dynamics will be defined by a balance between selection, mutation, and genetic drift.

Inbreeding and selection are likely to be involved in generating the overall long-term evolutionary response of starvation resistance. In fact, a significant decrease in female starvation resistance was observed for NW populations after 86 generations in the laboratory (see chapter 2). NB starvation resistance also showed a decrease between generations 94 and 184 (see chapter 3). This corresponds to a decrease of about 0.7 - 1.2% in female starvation resistance for every one per cent increase in the inbreeding statistic F, assuming an effective population size (N_e) around 300-500. These values are similar to those obtained in other studies of inbred *Drosophila* populations (e.g. 0.96%

in Ehiobu *et al.,* 1989; 0.7% in Rodríguez-Ramilo *et al.,* 2004). Therefore, inbreeding depression appears to play an important role in the long-term evolutionary patterns of starvation resistance.

Overall, it seems likely that a balance between the accumulation of recessive deleterious alleles and the countervailing effects of selection can explain the long-term evolutionary patterns observed (see Lande *et al.,* 1994; Charlesworth & Charlesworth, 1999; Reed & Bryant, 2001; Rodríguez-Ramilo *et al.,* 2004; Meffert *et al.,* 2006). The outcome of this balance will depend not only on the strength of selection on each particular trait but also on several other factors such as the specific genetic composition of each population, the average effect of deleterious mutations, population size, and the number of generations of confinement (Falconer & Mackay, 1996; Keller & Waller, 2002; Meffert *et al.,* 2006).

Repeatability of Laboratory Evolution

This study found general repeatability of evolutionary patterns. In fact, all experimental populations studied presented an increase in performance for fecundity related traits, while showing unclear evolutionary patterns for starvation resistance. As for the specific evolutionary dynamics involved in each adaptive process studied, the results obtained indicate clear variation in evolutionary rate, due to the significant impact of contingent factors. The influence of both temporal and spatial effects affecting the genetic variation and composition of founder populations and, therefore, the subsequent adaptation to the laboratory environment were clearly demonstrated (see chapter 2 and 4). In chapter 2 an analysis of the initial adaptation dynamics of the AR and TW populations derived from different natural locations suggests an impact of the wild source population on the evolutionary dynamics of laboratory adaptation during the first 15 generations.

In chapter 4, a broader analysis supported the relevance of both geographical effects (different wild source populations) and/or temporal effects of sampling (foundations from the same wild source population in different years) in the subsequent evolutionary dynamics in the laboratory. Differences in the initial genetic composition of the founder populations are the most likely explanation. However, interactions between the selective process and random genetic changes during laboratory culture cannot be completely ruled out (e.g. Cohan, 1984; Cohan & Hoffmann, 1989; de Brito *et al.,* 2005), despite the relatively high census sizes (around 1000 individuals) and short

time interval in the laboratory (around 20 generations). Alongside the effects associated with different initial genetic composition, the process of colonizing a new environment might also lead to significant genotype-by-environment interactions due to an alteration of the relationship between a specific trait and fitness relative to its effect in the ancestral environment. This alteration would then produces changes in the subsequent adaptive response (e.g. Teotónio & Rose, 2001; Teotónio *et al.,* 2002).

These effects are even more striking considering that all the wild populations that were sampled are closely located in Portugal. This suggests that geographically proximate wild populations can differ in their genetic composition and variability (see also Parsons, 1970; McKenzie & Parsons, 1974; Cohan & Hoffmann, 1989). Therefore, it highlights the need for careful sampling procedures in order to avoid potentially confounding sources of both temporal and spatial genetic variation when collecting samples from wild populations, even from the same geographical location. (e.g. Sgrò & Partridge, 2000; Hoffmann *et al.,* 2001b).

Overall, these results clearly demonstrate the impact of contingency factors in the early stages of adaptive evolution. The importance of contingency in adaptive evolution has been highlighted in several *Drosophila* studies (e.g. Hoffmann & Cohan, 1989; Huey *et al.,* 2000; Teotónio & Rose, 2000; Rose *et al.,* 2005) and in *E.coli* (e.g. Travisano *et al.,* 1995), as well.

Joshi *et al.* (2003) have found that ancestry and past selective history are likely to have a transient effect in adaptive evolution in the face of selection and genomic reshuffling due to recombination, particularly for traits closely related to fitness. On the other hand, Bieri & Kawecki (2003) did not find convergence to the same life-history phenotype in populations of the seed beetle *Callosobruchus maculatus,* despite 120 generations of uniform natural selection. Detailed analyses of the long-term evolutionary trajectories of laboratory populations subjected to a uniform selective regime will help to clarify these issues.

Real time evolution *vs.* Comparative approach to study laboratory adaptation

The results obtained from assays of fecundity characters over entire evolutionary trajectories *vs.* comparative analysis at single points in evolutionary time suggest that the comparative method can generate useful evolutionary inferences for traits presenting a clear adaptive pattern. But this was not the case for starvation resistance, for which the

comparative method provided misleading results relative to the evolutionary trajectories observed for this trait (see chapter 3; see also Matos *et al.,* 2004). These differences may partly explain discrepancies between research teams in the evolutionary patterns reported for this particular trait (e.g. Hoffmann *et al.,* 2001b; Matos *et al.,* 2002; Griffiths *et al.,* 2005; see details in chapter 3).

Genetic Diversity and Demography during Laboratory Adaptation

This study showed considerable genetic variability in the laboratory populations studied, as inferred from the analysis of ten microsatellite loci. NW populations presented an expected heterozygosity of 0.74 after almost 50 generations in the lab. This corresponds to retaining about 87 to 89% of genetic diversity, relative to the measured initial TW heterozygosity, used as an estimate of initial genetic diversity in the laboratory (Simões *et al.,* 2007d / see chapter 5). After 40 generations of laboratory adaptation AR and TW populations retained, respectively, 95% and 90% of their initial genetic diversity (Simões *et al.,* 2007e / see chapter 6).

The observed decline in the genetic variability of these laboratory populations is an expected outcome of the loss of alleles due to genetic drift, a process that depends on the effective population size (Falconer & Mackay, 1996). This process is likely to be enhanced in confined populations, due to general smaller population sizes, as well as the absence of the gene flow that occurs in natural populations.

It is relevant to notice that the rate of heterozygosity decline was not constant through time. In fact, both AR and TW populations presented a higher rate of decline in genetic diversity in the initial 14 generations than that observed between generations 14 and 40. The slowing down of the rate of heterozygosity decline through time may be a result of a smaller effective population size during the initial generations of laboratory adaptation (see below), as well as the loss of rare alleles during the earlier phase of laboratory evolution.

The relatively modest drop in genetic diversity observed in our laboratory populations suggests that these populations have not been subjected to extreme bottlenecks in their laboratory evolution. The careful maintenance and overall high census sizes (averaging around 900 individuals per generation) in our populations may explain these results. This is of particular interest considering that our populations were not subjected to any active management strategy, such as equalisation of individual

contributions (e.g. Frankham *et al.,* 2000; Gilligan *et al.,* 2005; Rodríguez-Ramilo *et al.,* 2006). In fact, we obtained lower rates of genetic variability decline per generation than those observed in studies of *D. melanogaster* populations maintained under active management procedures, though smaller effective population sizes (e.g. Montgomery *et al*., 2000; England *et al*., 2003) – see chapter 5.

As expected, heterogeneity in allele frequencies increased between populations during laboratory culture due to random genetic drift, resulting in higher F_{st} values, both between replicate populations derived from the same foundation and between populations of different foundations. For example, the average pairwise genetic differentiation between AR and TW populations was 0.016 at generation 3, 0.071 at generation 14 and 0.106 at generation 40 (see chapter 6).

An overall similarity in genetic differentiation patterns was observed between the different sets of populations through time. Also, the genetic differentiation between replicate populations within each set of populations did not differ significantly in the 3 independent foundations (NW: F_{st} =0.104, C.I= 0.075; 0.134 at generation 49; AR: F_{st} =0.064, C.I.=0.044; 0.083 and TW: F_{st} =0.078, C.I.=0.051; 0.107 both at generation 40; see chapters 5 and 6).

N_e/N ratios (ratios between effective population size and census size) were also similar between different foundations (AR, TW and NW), although generally fairly low (average estimate of 0.27 for both NW and TW populations; 0.30 for AR populations). These low values are most likely due to unequal family contributions since, as reported above, no severe bottlenecks occurred in the evolution of these laboratory populations (see chapter 5 and 6).

Taken together, these results suggest an overall repeatability in the evolutionary dynamics of microsatellite loci during laboratory adaptation across different independent foundations, regardless of location and year of sampling. It is worthwhile noting that, despite the different evolutionary patterns and prior history of our laboratory populations, the overall impact of genetic drift on genetic diversity and differentiation during laboratory evolution appears to be quite similar across foundations.

Despite this overall similarity, a comparison of the genetic parameters obtained from the two sets of populations evolving in synchrony (AR and TW populations) suggests that drift effects have been slightly more pronounced in the TW populations. Furthermore, lower effective population sizes (N_e) were observed in the TW

populations. A possible explanation for these slight differences between AR and TW populations resides in the fact that AR populations have shown a slower adaptive rate compared to TW populations, particularly for the first 14 generations of laboratory adaptation (see chapter 2). The stronger effects of selection in the TW populations could thus partly explain their lower effective population size and, therefore, the higher drift effects observed.

Our temporal genetic analyses also support an effect of selection on the overall population demography. Significant changes in the N_e/N ratios during laboratory adaptation were found, with N_e values being lower during the first period of adaptation (generations 3 to 14) relative to the second period (generations 14 to 40), particularly in TW populations. The most likely cause for this is the impact of early adaptation to laboratory environment, which contributes to lower initial N_e values as a result of greater variation in reproductive success within a population at each generation, even if census size (N) is high, thus leading to low N_e/N ratios (Hedrick, 2005; Noruma, 2005). This effect is likely to be greater in the early stages of adaptation (see chapter 6). A possible relationship between neutral genetic and adaptive patterns during laboratory evolution will be further addressed below.

Does molecular variation reflect evolutionary potential?

A recent meta-analysis by Reed & Frankham (2001) has shown weak correlation between molecular variation and quantitative variation. Since the latter is directly involved in adaptive processes, the authors concluded that molecular markers do not accurately reflect adaptive evolutionary processes (e.g. adaptive differentiation) or evolutionary potential. Several factors can be involved, such as predominant effects of selection in quantitative genetic variation *vs*. effects of genetic drift on generally neutral molecular markers, non-additive genetic variation in quantitative traits and/or sampling variation in the estimates (Reed & Frankham, 2001; McKay & Latta, 2002).

More recently, Reed & Frankham (2003) have found a significant positive correlation between molecular heterozygosity and population fitness. According to the authors, this correlation is due to the mutual dependence of quantitative genetic variation and heterozygosity on the effective population size. This might be particularly relevant in small populations, with inbreeding and drift undermining population fitness, thus generating a positive correlation between molecular variation and fitness due to population size effects. As a corollary, it seems plausible that small populations will

lack standing genetic variability for fitness-related traits. Thus, though not saying it literally, the authors have somehow retreated relative to their previous statement (Reed & Frankham, 2001 cf. Reed & Frankham, 2003), which reveals how this field is still lacking accurate empirical studies.

A possible relationship between neutral genetic variability and evolutionary potential was tested in our study, based upon the results obtained for initial genetic variability measured with molecular markers (at generation 3) and the subsequent evolutionary response of life-history traits in both AR and TW populations. This study did not find any consistent association between neutral genetic variability and adaptive potential. In fact, while different adaptive dynamics were found to occur between AR and TW populations, with TW populations evolving faster than AR during the first 15 generations of laboratory culture (see chapter 2), this did not correspond to any significant difference between AR and TW variability levels: the AR and TW neutral variability at generation 3 was remarkably close (H_e=0.825 for AR; H_e=0.834 for TW) – see chapter 6. Thus, neutral genetic variability patterns of both AR and TW were uninformative in respect to the ability of these populations to adapt to a new environment. These results highlight the point that genetic variation for fitness traits cannot be accurately determined from levels of molecular genetic variability.

Independently from this lack of association between initial neutral genetic variability and evolutionary potential, one might expect an association between adaptive dynamics and temporal decline of molecular heterozygosity. In particular, a lower N_e is expected in populations evolving faster, due to an increase in the variance of family contributions, leading to a greater loss of genetic variability. This would translate to significantly higher genetic variability for AR relative to TW populations either after 14 or 40 generations of laboratory adaptation. Though our results did not support such a prediction - no significant differences were found between AR and TW genetic variability either at generation 14 or 40 - it is worth noting that a tendency was observed in the expected direction, since TW populations presented lower genetic variability than AR populations in those generations. It is likely that the magnitude of differences in the selective response between the AR and TW populations was not enough to generate conspicuous differences between these populations at the molecular genetic level. This effect could also have been inflated by the initial similarity in variability patterns in both sets of populations. An increase in statistical power should help to test more

accurately this type of interaction between genetic drift and adaptive dynamics, such as would arise with populations that differ more in their initial genetic diversity.

On balance, these results do not support any clear association between the adaptive processes occurring in our laboratory populations and the overall patterns of variability presented by molecular markers. This adds to a growing body of data supporting the need for caution when using molecular markers to infer evolutionary potential and adaptive differentiation between populations (e.g. Hedrick, 1999; Reed & Frankham, 2001; McKay & Latta, 2002; Edmands & Harrison, 2003; Knopp *et al.*, 2007).

Can we infer the evolutionary processes underlying population divergence by comparing quantitative and molecular differentiation?

Using the prior knowledge of the evolutionary history and patterns presented by our populations, this study addresses the importance and accuracy of predictions based on combined quantitative and molecular data (Q_{st} *vs.* F_{st} approach) in the study of evolutionary differentiation. It can be concluded from the analysis presented here that this approach can generate inferences about the importance of directional natural selection in population differentiation.

Quantitative differentiation for fecundity traits between generations 3 and 40 of laboratory culture was generally higher than neutral genetic differentiation between the same generations. This result was observed for both AR and TW populations. As such, our a priori expectation of directional selection acting on fecundity traits ($Q_{st}>F_{st}$) during laboratory culture is corroborated by the Q_{st} *vs.* F_{st} approach (see chapter 6).

It is not likely that these results are due to non-additive and/or environmental effects that may have affected the quantitative genetic differentiation estimates, as a result of the use of phenotypic data rather than direct additive genetic variance. Recent studies have concluded that $Q_{st}>F_{st}$ results are unlikely to be caused by either dominance (Goudet & Büchi, 2006) or epistasis (Whitlock, 1999; López-Fanjul *et al.*, 2003). Also, in order to account for possible environmental effects, the estimation of the within population variation was based on an assumption of the heritability in life-history traits (see chapter 6). Furthermore, considering the experimental design used in our assays, micro-environmental effects are most likely to inflate the within-population variance component thus making the $Q_{st}> F_{st}$ finding rather conservative.

The results presented here concur with those obtained by other studies using controlled laboratory experiments to address the Q_{st} *vs.* F_{st} approach. Porcher *et al.* (2004) demonstrated that Q_{st} increased with selection heterogeneity (related with the variance in the intensity of selection between populations) when genetic drift is limited. Morgan *et al.* (2005) used laboratory populations subjected to selection for wheel-running activity to analyse quantitative and molecular differentiation and concluded that the Q_{st} *vs.* F_{st} comparisons generally produce the correct evolutionary inference.

These are relevant findings, since $Q_{st}>F_{st}$ has typically been observed in other studies comparing these differentiation estimates in natural populations of unknown prior evolution (e.g. Koskinen *et al.*, 2002; see Merilä & Crnockrak, 2001 for a review). These empirical results reinforce the interpretation of a prominent role of natural selection in promoting phenotypic divergence (e.g. see Merilä & Crnockrak, 2001; McKay & Latta, 2002).

Taken together, these results support the interpretation that adaptive and neutral differentiation are apparently decoupled. Although directional selection leads to both phenotypic and molecular differentiation, the former is likely to occur at a much higher rate than the latter (McKay & Latta, 2002).

The results obtained here also highlight some of the potential downfalls of dealing with variance component ratios, such as wide confidence intervals that can obscure evolutionary inferences (see chapter 6).

Detecting natural selection with molecular data

Although the information obtained with molecular markers generally reveals the effects of genetic drift and neutral evolution primarily, it is possible that positive selection affects particular microsatellite *loci*. This is most likely to occur through genetic hitchhiking, due to linkage disequilibrium between a positively selected region (or gene) and the neutral loci (Maynard Smith & Haigh, 1974; Barton, 2000), leading to a decline in genetic variability at the microsatellite loci and its flanking regions - a selective sweep.

This study reports evidence suggesting that such a selective sweep event occurred in the region of one of the ten microsatellite *loci* examined – *Dsub14* - during the first 14 generations of laboratory adaptation (see chapter 6). Furthermore, the detected deviation from drift expectations was due, in all three TW populations, to an

increase in frequency of the same allele, one that was not the most frequent at generation 3. This pattern is difficult to explain in terms of a random genetic mechanism. However, this allele did not increase in frequency between generations 14 and 40 in these populations, as might be expected by directional selection. In fact, continued action of such directional selection in laboratory populations of moderate size should instead have led to a fixation of this allele, unless linkage disequilibrium between the selected region and the molecular marker was broken by recombination.

An alternative possibility is the maintenance of genetic variability at the linked locus due to the effects of balancing selection, which may occur in the adaptive process after an initial period of consistent directional evolutionary change (e.g. Hansson & Westerberg, 2002; Ferreira & Amos, 2006). Both these observations at a later generation and the not so clear temporal pattern of allele frequency changes of AR populations (though also giving significant deviations from neutral expectations in this locus) leads to some reservations as to whether in fact a selective sweep has been involved. Studies involving more generations, as well as independent foundations, will be important to further our understanding of the evolutionary dynamics involving this microsatellite variant.

The fact that the locus in question (*dsub14*) is located in the O chromosome is relevant, because of the high frequency of inversion polymorphisms that may prevent extensive recombination in this chromosome and, therefore, favour hitchhiking events between positively selected regions and molecular markers (Hoffmann *et al.,* 2004; Munté *et al.,* 2005). Furthermore, the low level of genetic polymorphism shown by this particular microsatellite locus is consistent with its location in a genomic region previously targeted by natural selection. A similar scenario was observed by Catania & Schlötterer (2005), who described a partial selective sweep occurring in a microsatellite locus showing reduced levels of polymorphism in one European population of *Drosophila melanogaster*.

To identify the genomic region affected by this potential selective sweep, further research should test for low polymorphism in the surrounding genomic region, through the analysis of adjacent microsatellite loci and/or sequencing of flanking regions about the locus in question. This might identify candidate genes directly involved in adaptation.

Other recent studies have also found evidence for the occurrence of selective sweeps using microsatellite data, particularly in *Drosophila* (Harr *et al.,* 2002; Kauer *et*

al., 2003; Catania & Schlötterer, 2005). Although taken with caution, given the small number of loci sampled, our results favour the interpretation that natural selection can often affect the *Drosophila* genome, either through hitchhiking events or to direct regulatory function of non-coding DNA sequences (cf. Andolfatto, 2005).

7.1 Final Considerations

I will now briefly highlight some of the most relevant points of this study:

Adaptation to the laboratory environment enhances fecundity traits. Clear evolutionary improvement in fecundity-related traits was found as a consequence of introduction to a novel environment in all populations analyzed. As such, adaptation to the laboratory environment was not constrained by lack of genetic variation in any of the different populations. The magnitude of the evolutionary response was positively correlated with the disparity between the performance of the experimental population immediately upon laboratory introduction and that of a control population.

Simple correlated responses to adaptation did not prove accurate. While increased performance for all life history traits could be expected during the early stages of laboratory adaptation, the results presented here show varied evolutionary patterns among the several life-history traits measured. For example, a lack of consistent directional evolutionary response was found for starvation resistance, development time, and viability in the populations studied, while other traits showed overall relevance (fecundity related traits). The relative contribution of different traits to fitness in the novel environment is likely to be highly variable, independently of their possible role in the ancestral, natural environment.

Evolutionary responses to the laboratory environment are qualitatively uniform while varying quantitatively across foundations. General repeatability of evolutionary response across populations studied was found, though the specific adaptive dynamics (e.g. rate of convergence to control populations) differed across foundations, as a function of either temporal or spatial effects.

Long-term domestication involves a balance between opposing forces of selection and inbreeding. As *Drosophila* populations adapt to the novel laboratory environment, they

appear to approach an evolutionary equilibrium, as suggested by the slowing down of evolutionary change in the long-established populations studied here. This may be due to depletion of additive genetic variability for fitness-related traits. Under these circumstances, selection is likely to diminish in effect and the impact of inbreeding may prove greater. Inbreeding may be particularly relevant in shaping the long-term laboratory evolution of adult traits less relevant to fitness in the laboratory environment (e.g. starvation resistance).

Considerable molecular genetic variability can be retained in laboratory populations. Our populations showed high levels of molecular genetic variability, even after 40 - 50 generations in the laboratory, with a loss of just 5 to 13% of their initial genetic variability. This suggests that careful maintenance – such as high census population sizes and prevention of non-random mating - of captive populations is likely to successfully avoid the loss of considerable levels of genetic variability, even in the absence of direct management of individual contributions.

It is relevant to notice that the rate of decline in genetic variability slowed through time, with correspondingly higher effective population sizes relative to those of the initial generations. This is probably the result of the higher selective pressures experienced by the laboratory populations in the initial generations immediately after foundation.

Molecular measures of genetic diversity do not accurately reflect differences in the evolutionary potential of populations adapting to the laboratory environment. Patterns of molecular genetic diversity during laboratory adaptation showed an overall similarity across different foundations, despite differences in early adaptive dynamics; as such, measures of genetic variability of molecular markers do not allow straightforward inferences of evolutionary potential.

The combined use of quantitative and molecular genetic information provides valuable insight into the evolutionary history of populations. The use of molecular data under the null hypothesis of neutrality allows us to address the relative importance of genetic drift and selection in driving population divergence through the use of Q_{st} *vs.* F_{st} comparisons. Furthermore, neutral molecular markers provided valuable information on population history and demography;

Natural selection affects molecular genetic diversity through hitchhiking. This study suggests a possible selective sweep at one of ten microsatellite loci during the first 14 generations of laboratory adaptation. However, further analyses of this particular locus in subsequent generations, as well as the sequencing of flanking regions, will allow us to test the consistency of this indication.

7.2 Future Research

Experimental evolution is a field that is growing rapidly, and successful examples of its applications mount (see Prasad & Joshi, 2003; Chippindale, 2006). An increasing number of evolutionary biology studies have been addressing the genetic basis of observed phenotypic changes. However, we are far from fully understanding the intricate relationship between genetic and phenotypic change during evolution. Our understanding of the connection between evolution at the phenotypic and genotypic levels will certainly improve with the application of techniques of molecular genetic analysis in an experimental evolution framework (e.g. Pletcher *et al.,* 2002; Hoffmann *et al.,* 2003; Morgan *et al.,* 2005; Laayouni *et al.,* 2007).

This study follows this path, combining both phenotypic and genetic information to characterize an ongoing adaptive process. Here a microsatellite screening of an adaptive process is presented, including both ancestral and derived laboratory populations. However, the number of microsatellite loci used in this study does not allow a complete survey of the genome of the adapting populations. A wide microsatellite screening of the genome of populations experiencing an adaptive event would increase the possibility of detecting selective sweeps, and construct a detailed hitchhiking map (Harr *et al.,* 2002; Schlötterer; 2002a,b). As a complement, the analysis of linked microsatellite loci in the vicinity of a candidate genomic region will allow us to validate the inference of a selective sweep and thus reduce the number of false positives identified in initial genomewide surveys (Wiehe *et al.*, 2007). This would be a crucial step in the characterization of candidate genes and genomic regions involved in adaptation.

Furthermore, the advent of microarray technology allows the study of genome-wide expression profiles, an approach that can provide valuable insight in evolutionary studies (Gibson, 2002; Ranz & Machado, 2006). Microarrays measure the level of

messenger RNA abundance in thousand of genes through hybridization with DNA probes (complementary or oligonucleotide arrays). The combination of microarray technology and quantitative genetics allows us to address in detail the molecular genetic basis of trait variation as well as identifying candidate genes (Jansen, 2003). Candidate genes can be discovered through transcriptional differences associate with trait variation. In fact, quantification of gene expression profiles for lines differing in the expression of a certain trait will allow the detection of the loci involved in quantitative variation (Jin *et al.,* 2001; Gibson, 2002). Thus, microarray technology can be a powerful tool with which to analyze the genetic architecture of complex traits (e.g. Toma *et al.,* 2002). Also, microarray experiments appear to be sensitive enough to address within-population genetic variation (e.g. Bochdanovits *et al.,* 2003), thus allowing direct measurement of variation in fitness related traits (see Drnevich *et al.,* 2004).

The study of gene expression patterns at different development stages would also allow the investigation of the triggering of the genetic pathways involved in development (Gibson, 2002). It should thus ultimately allow us to understand the impact of developmental and physiological pathways on the expression of relevant fitness traits during an evolutionary process, by detecting changes in the temporal profiles of gene networks as populations undergo an adaptive event.

Overall, this study highlights the benefits that can arise from the combination of experimental evolution studies with molecular approaches to characterize in greater detail the phenotypic and genetic changes during an adaptive event. It also shows the importance of studying evolutionary dynamics in captivity to better understand *ex situ* conservation programs. At the molecular level, it raises the possibility of further studies investigating the particular genomic regions involved in local adaptation.

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